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Attention: M Weaver - Examiner

Your reference	Our reference	Date
	PL977PCT/MCG/KOK	16 February 2000

Dear Sir,

Re: International (PCT) Patent Application No.PCT/IE99/00012 of NATIONAL UNIVERSITY OF IRELAND, CORK

We refer to the written opinion dated 19 November 1999.

The Examiner states that Claim 1 is directed to a method for the diagnosis of pre-eclampsia (PE), eclampsia or intrauterine growth retardation (IUGR). The Examiner goes on to argue that D1 describes low levels of HLA-G exon 2 and 3 polymorphism in an inbred population, which shows no deleterious effects on the pregnancy outcome. D2 discloses reduced expression of HLA-G1 protein in term placenta from PE pregnancies and suggests that decreased HLA-G protein expression, whether by mutation or polymorphism in the HLA-gene may lead to foetal loss, IUGR or PE. The Examiner believes that similar conclusions of an association between reduced HLA-G expression and PE are to be found in D3, D4 and D5. In addition, D6 discloses an association between PE and the placental expression of variant allele of HLA-G. The Examiner concludes that the clear and unambiguous teaching of all of these documents is that differences in HLA-G expression are associated with a number of abnormal pregnancy states, in particular PE and IUGR. The Examiner believes it would be a straight forward matter, devoid of any inventive merit, for the skilled person to apply the collective teaching of these documents to solve the problem of providing a means of determining susceptibility to any of the abnormal pregnancy conditions, such as PE, mentioned in D1 to D6. The Examiner's conclusion is that Claim 1 lacks inventive step.

The Applicant would argue that the present application shows that both paternally inherited and maternally inherited HLA-G polymorphisms are linked to normal pregnancy outcome and that the paternal and maternal effects are different. The present invention shows that transmission of HLA-G alleles to PE offspring is distorted. This is not obvious from any of the prior art documents and is both completely novel and unexpected. Without the present invention, it is not possible to diagnose PE susceptibility. There is no disclosure in the prior art that there is linkage of HLA-G to normal pregnancy outcome and no evidence that allele transmission of HLA-G to PE offspring is distorted.

The present invention demonstrates linkage of HLA-G to normal pregnancy outcome. This is novel. For example, D6 shows an association between HLA-G and PE. Association between an allele and a disease does not mean that the associated gene causes the disease, but it does indicate that there is a risk factor of some degree. By contrast, linkage provides direct evidence for a role of a genetic locus in a disease. For example, red hair is associated with freckles. However, red hair does not cause freckles. If red hair were linked to freckles, then the red hair gene or a gene close by would play a direct role in causing the freckles. In the present invention, the Applicants demonstrate linkage between HLA-G and normal pregnancy outcome. The Applicants have also shown that transmission of HLA-G alleles to PE offspring is distorted. This means that HLA-G plays a direct role in normal pregnancy outcome and PE. HLA-G can thus be used for diagnosis of susceptibility to normal pregnancy outcome and PE. If the invention only showed association, then it would only be possible to predict risk, and not to identify susceptibility. To those skilled in the art there is a considerable difference between linkage and association.

If one takes the prior art as a whole, there have been multiple reports in the literature of association between PE and numerous other genes and proteins (see enclosed listing). A survey of Medline shows up there are 305 hits for a search on PE and association. For each of these reports, one can speculate that polymorphisms in the relevant gene causes PE. However, using association or direction measurement of RNA, protein or the like, it is not possible to say whether the result is cause or effect. For example in PE it is known that trophoblast invasion is shallow so that measurement of many trophoblast RNAs, proteins or protein activities will be disturbed. This does not mean that this is the cause of the disease and does not identify a gene or protein target for therapy. Thus the plethora of PE association studies in the literature has identified numerous candidates, such as HLA-G, that can be considered potential candidates. However none of the prior art to date demonstrates that HLA-G actually plays a causative role in PE.

Furthermore all of the prior art studies have concentrated on the influence of the mother, looked at the placenta for gene mRNA and protein levels, or looked at the offspring. Most have focused on the association between the maternal genotype and PE. The present inventors have addressed the fact the foetal genetic profile may be central to the disease, and therefore considered the paternal genetic component to be equally important to the maternal genetic component in disease manifestation.

The Examiner has cited D1 to D6 against the invention;

D1 describes 9HLA-G alleles and striking linkage disequilibrium between HLA-G and HLA-A alleles, which were studied in the Hutterites. On page 120, first complete paragraph, the low levels of polymorphism in both exons 2 and 3 in the Hutterites is stated not to have had obvious deleterious effects on pregnancy outcome, because this population is among the most fertile human population ever studied. Furthermore the population has much lower incidences of recurrent miscarriage, pre-term labour, IUGR, PE or other complications in pregnancy, compared to the general population. The paper goes on to conclude that limited polymorphism and HLA-G iso forms, per se, is not likely to be associated with adverse pregnancy outcome and the document speculates that other factors may effect PE, miscarriage or the like. Thus this document leads one away from the present invention, as the above quoted statement is exactly the opposite of the present invention. The Examiner argues that the clear and unambiguous teaching of this document (among others)

is that differences in HLA-G expression are associated with PE and IUGR. In fact, the polymorphisms studied in the paper had no deleterious effect on pregnancy, which is exactly the opposite of the Examiner's argument.

D2 discloses a large number of polymorphisms in the HLA-G gene. There is speculation that reduced expression of HLA-G proteins may be associated with poor placental invasion (see Section 4.2), but the document goes on to state that it is not possible to determine from the study whether the reduced expression of HLA-G at term was a cause or a result of the pre-eclamptic state. Thus the document does not disclose whether or not HLA-G is cause or effect in PE or IUGR. In the conclusion (Section 6) the authors go on to state that "HLA-G remains an intriguing gene and that our current limited understanding of the function and evolution of the gene warrants further investigation." Thus this document merely speculates and indicates that further investigation of the role of HLA-G in pregnancy is required. The present invention actually shows linkage between HLA-G and normal pregnancy outcome and could not be considered obvious in the light of a document that states it is still necessary "to learn about the function of HLA-G1 proteins in a biological context."

D3 is a review of immunological aspects of normal human pregnancy and clinical reproductive abnormalities, including pre-eclampsia, premature ovarian failure, endometriosis and recurrent abortion. In its discussion of the immuno biology of the pre-eclampsia- eclampsia syndrome (see second column, page 12) is a study by Lim et al is described which combined in vitro and immunohistochemical analysis to show that trophoblasts from patients with pre-eclampsia failed to express HLA-G mRNA and protein. Also described is a HLA-G deletion polymorphism among families of normal subjects and those with a history of pre-eclampsia. However HLA-G genotypes and deletion frequencies were found to differ among the study groups. "Thus, reduced expression of HLA-G is associated with pre-eclampsia, but whether this is due to aberrant trophoblast differentiation in this condition, or as the result of a structural gene mutation has not been elucidated". Thus again there is speculation about the role of HLA-G but no conclusive evidence as to what that role might be or evidence of a linkage between the gene and PE. Thus this document does not disclose the present invention. In fact the paper cited in D3 by Humphrey et al (ref 95) describes a population association study of HLA-G genotypes from pre-eclamptic and eclamptic patients and control groups. Humphrey et al concluded the "no detectable relationship between susceptibility to PE or being born of a PE pregnancy and HLA-G genotype could be demonstrated". Again this is completely the opposite of the finding of the present invention. This is a particularly significant finding since the deletion polymorphism of the Humphrey study was the same as that used in the present invention.

D4 reports the analysis of RNA from PE and normal placentae. The document discloses a reduction in the ratio of TGF beta 1 and HLA-G RNA and PE placenta compared to normal placenta. The conclusion is that transcription of TGF beta 1 and HLA-G and /mRNA stability is reduced in placenta from patients with PE and suggests that "reduced expression of these immunosuppressive factors involved in placental invasion, may have a pathogenic role in human pre-eclampsia". Again this document proposes a cause for PE but does not show a linkage between HLA-G and PE or IUGR.

D5 reports on the study to determine whether or not the expression of HLA-G protein on the trophoblast is altered in pre-eclampsia, and shows that there is reduced

expression of HLA-G protein on the extravillous trophoblast and PE placenta, relative to a normal placenta. However the document concludes that the "the attenuated expression of HLA-G protein on the extravllious trophoblasts could be at play in the pathophysiology of pre-eclampsia". Again this document simply speculates on an association between HLA-G and pre-eclampsia.

D6 describes a study which investigated whether or not trophoblast expression of a recently identified polymorphism of HLA-G was associated with a clinical syndrome of PE. Placental biopsies were examined by RNA analysis and the study concluded that there is a strong association between the HLA-G allele (A and code 110) and PE. Again this document proposes association and not linkage.

Thus Applicants submits that none of the prior art documents shows a linkage between HLA-G expression and PE or IUGR. It is not at all obvious that because there is association between a gene and a disease, that there is linkage between them. Thus Claim 1 does not lack inventive step.

The Examiner has raised similar objections to Claim 23, which is directed to a kit for diagnosis of susceptibility to a normal pregnancy, PE, IUGR or miscarriage. Such a kit cannot be obvious, unless it has previously been shown that there is linkage between HLA-G and PE or IUGR, which as described above is not the case.

The Examiner has also raised objection to Claim 16 which relates to a method for screening for potential therapeutic agents by comparing the expression level of a gene or protein in specified cells following interaction with HLA-G or a HLA-expressing cell and that of expression levels in normal pregnancy, PE or IUGR or miscarriage or miscarriage related infertility. As described above a known association of differences in HLA-G expression is not sufficient to render obvious such screening methods. Accordingly Applicants submits that Claim 16 does involve an inventive step.

The Examiner also argues that Claim 18 lacks inventive step because as HLA-G is known and it would be an obvious matter for the skilled person to formulate an amount of HLA-G in a composition which would be appropriate for the intended pharmaceutical application. However, the prior art does not show that HLA-G is directly involved in susceptibility to be PE and that HLA-G is defective in PE. The present invention is the first to show that this is the case, thus pointing to the use of HLA-G as a therapeutic agent. Since the prior art only shows an association between HLA-G and PE there is no reason in the prior art to suppose that HLA-G treatment would be effective. Since at least two of the cited papers teach completely away from the present invention, it could not be obvious to use HLA-G as a therapeutic agent.

The Examiner has also raised an objection that Claim 24 lacks an inventive step. Claim 24 relates to the use of DNA sequence selected from any one of sequence ids 1 to 21 for the diagnosis of susceptibility to, or in a test kit for the diagnosis for susceptibility to, normal pregnancy, PE, eclampsia, IUGR, miscarriage or miscarriage-related infertility. The Examiner argues that the entire gene sequence of HLA-G is acknowledged to be known as are polymorphisms of exons 2, 3 and 8. Again the Examiner argues that given the known association of these polymorphisms with miscarriage, pre-term labour and other pregnancy complications, it would be obvious for the skilled person to use nucleic acid sequences

which are able to detect such polymorphisms, when assessing susceptibility to such conditions. As discussed above, D1 and D2 did not find any association between the C/T 93 polymorphism or the I/DE8 polymorphism with PE. D6 did find association but only with the single polymorphism 110, which is not included in the relevant claim. Likewise the I/DE8 polymorphism has been shown not to be associated with PE in the prior art (Humphrey et al which is referred to D3). One cannot therefore derive from the prior art that there is a known association of these polymorphisms with pregnancy complications. D1 and D2 speculate on the impact of the polymorphisms on protein expression that may impact on PE, but D2 clearly states that this cannot be determined from their work. Applicant therefore submits that Claim 24 is inventive.

The Examiner also goes on to raise objection to certain terms in Claims 1, 4 and 11 which the Examiner considers are not supported by any concrete evidence in the form of examples which would allow the skilled person to reproduce the alleged invention.

1. "HLA-G linked nucleic acid sequence". It is well known in the art that linkage disequilibrium exists across the whole HLA locus covering some 4 million base pairs. Thus if HLA-G is found to be linked to a disease by showing that a polymorphism is in disequilibrium with the disease, this could mean that HLA-G is causative or is linked to a gene that is causative of the disease. This latter gene must be physically linked to the HLA-G as linkage disequilibrium does not extend over large areas of the chromosome. To one skilled in the art it is clear that the HLA-G is causative of PE or is closely linked to a gene which causes PE. The present invention makes it obvious that one could use polymorphisms in genes linked to HLA-G for diagnosis of PE, as well as polymorphisms in HLA-G itself. Applicant submits that someone skilled in the art would not find this term at all unclear and would not require any further support, in terms of examples, to reproduce the invention. Similar arguments apply to the terms "proteins encoded by HLA-linked genes" and "mRNA transcribed from HLA-G linked genes".

The Examiner has also raised objection to use of the terms "and/or" and "or" in the claims, and considers that their use renders the claims unclear and and makes it difficult to determine the scope of the claim. Applicant would contend that this is not at all unclear to a person skilled in the art. Because of linkage disequilibrium it would be apparent to the skilled person in this art that HLA-G nucleic acid or a HLA-G -linked nucleic acid could be used in the invention. Similarly the HLA-G protein or protein encoded by a HLA-G linked gene could similarly be used. Thus is it legitimate for the Applicant to use the terminology "and/or" to cover the claimed invention. The fact that a claims covers a large number of embodiments does not of itself make a claim unclear and the wording is a legitimate attempt to protect the invention which the Applicant has made available to the public.

Claim 19 has been objected to by the Examiner as merely stating what is desired to be done without indicating, in terms of technical method features, how this is to be achieved. Claim 19 has now been redrafted to relate to use of HLA-G in a method for screening potential PE or IUGR, or miscarriage or miscarriage related infertility theraputications, which we believe overcomes the Examiner's objection.

The Examiner also raised objection to the use of the term "effective HLA-G" in part (a) of Claim 20. The word "effective" has been deleted from this claim.

Finally we enclose herewith a list of 10 abstracts which show association between PE and genes other than HLA-G come in support of the Applicants contention that the prior art as a whole does not show linkage between a PE and HLA-G. Also enclosed are amended pages 60, 61 and 62, in triplicate. A form 1037 is also enclosed for confirmation of receipt of this letter.

Yours faithfully,

Tomkins & Co.

inhibitors of cytokines and/or tumour necrosis factor alpha and/or derivatives of cytokines and/or tumour necrosis factor-alpha, optionally with pharmaceutically-acceptable carriers or excipients.

19. Use of HLA-G or HLA-G expressing genes in a method of screening for potential therapeutic agents for the treatment of a condition selected from:-. pre-eclampsia, eclampsia, intrauterine growth retardation, susceptibility to miscarriage and miscarriage-related infertility.

20. A method for the prevention of pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility selected from:

a) treatment of a female with all or part of a pharmaceutically effective amount of a HLA-G and /or peptides which bind to HLA-G and / or cells expressing HLA-G;

b) treatment of a female with all or part of a pharmaceutically effective amount of molecules or inhibitors of molecules whose level or activity is directly or indirectly altered by HLA-G action;

c) treatment of a female with all or part of a pharmaceutically effective amount of molecules which inhibit the interaction between HLA-G and one or more of its receptors;

d) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters HLA-G expression;

e) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters HLA-G related blood mononuclear cell activity;

f) treatment of a female with all or part of a pharmaceutically effective amount of an agent which mimics all or part of HLA-G action;

g) treatment of a female with blood mononuclear cells that recognise foetal and / or self HLA-G;

h) treatment of a female with HLA-G and / or cells expressing HLA-G or variants thereof;

i) treatment of a female with one or more antibodies which bind to HLA-G and / or cells expressing HLA-G and / or any receptor for HLA-G;

j) introduction of one or more variants of the HLA-G gene and /or its receptor into a female and / or male;

k) introduction of an inhibitor of expression of the HLA-G gene and/or its receptor into a female and/or male;

l) inactivation of one or more variants of the HLA-G gene and/or its receptor in a female and/or male.

21. A method for improving pregnancy success selected from:

a) pre-treating the female with sperm and/or attenuated forms thereof, and/or semen and/or fractions thereof from a male with a known HLA-G genotype, prior to mating with a male of a different HLA-G

genotype, and/or in vitro fertilisation using sperm from a male of a different HLA-G genotype and/or embryo transfer where the male HLA-G is of a different HLA-G genotype;

b) mixing sperm of a known HLA-G genotype with sperm and/or attenuated forms thereof, and/or semen and/or fractions thereof from a male with a different HLA-G genotype prior to in vitro fertilisation.

22. A method as claimed in claim 21 wherein fertility and / or pregnancy outcome are improved by selection of male and / or female partners and / or sperm and / or ova and / or recipients of fertilised eggs and / or zygotes / and / or embryos so that (a) their HLA-G and /or HLA genotypes and /or serotypes or (b) the activity of their HLA-G and / or blood mononuclear cells interacting with HLA-G are indicative of normal pregnancy outcomes and / or not associated with pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility.

23. A test kit for the diagnosis of susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility or for monitoring progress of pregnancy comprising:

a) oligonucleotide primers for amplification of all or part of the HLA-G gene and /or HLA-G linked DNA;

b) amplification reagents for amplification of genomic DNA and / or RNA segments, selected from a DNA / RNA polymerase, a reverse transcriptase, the deoxyribonucleotides dATP, dCTP, dGTP, dTTP and dUTP, and /or ribonucleotides ATP, CTP, GTP, TTP and UTP, and reaction buffer;

c) reagents for identifying sequence variants in DNA and / or RNA;

d) control DNA and /or RNA.

24. Use of a DNA sequence selected from any one of Sequence I.D.s 1 to 21 for diagnosis of susceptibility to or in a test kit for the diagnosis of susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility, for monitoring progress of pregnancy, for use in the manufacture of a medicament, in a method for screening potential therapeutic agents, in a method for screening for potential diagnostic indicators and/or drug targets, in a method for improving pregnancy success or in a method for the prevention of pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility, for monitoring progress of pregnancy.

25. A method for induction of tolerance in a host to a non-self tissue which comprises administering HLA-G and /or HLA-G loaded with peptides from the non-self tissue and /or HLA-G expressing cells derived from or related to the non-self tissue, and/or a non-self tissue bearing an introduced HLA-G so that HLA-G is expressed in all or part of the tissue.

26. A method for the treatment of autoimmune disease which comprises administering HLA-G and /or HLA-G loaded with peptides from a self and/or non-self tissue and / or with specific autoimmune antigen and /or HLA-G expressing cells from a self and/or non-self tissue and/or a self and/or self tissue bearing an introduced HLA-G gene so that HLA-G is expressed in all or part of the tissue.

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List of 10 abstract showing association between PE and genes other than HLA-G

1: Hum Genet 1999 Dec;105(6):641-7

Genetic susceptibility to pre-eclampsia and chromosome 7q36.

Guo G, Lade JA, Wilton AN, Moses EK, Grehan M, Fu Y, Qiu H, Cooper DW, Brennecke SP

Pre-eclampsia is the most common serious medical disorder of human pregnancy. The human endothelial cell nitric oxide synthase (eNOS) gene is a candidate forpre-eclampsia/eclampsia (PE/E) susceptibility. A linkage study was performed onAustralian PE/E families using 25 microsatellite markers from chromosome 7, oneof which (eNOS-CA) resides within the eNOS gene. No significant linkage wasfound for the eNOS-CA marker using either parametric or non-parametric analysis. However, D7S 1805 from the eNOS gene region on 7q36, gave a suggestion oflinkage using parametric analysis (maximum LOD score =2.143 at theta=0.14) and non-parametric APM analysis (T1/sqrt(p)=3.53; P=0.002). Further, an association study was performed on unrelated PE/E cases and controls from both Chinese and Australian populations to test for a relationship between the eNOS gene and PE/E. No association was found between the eNOS-CA marker and PE/E in either population. However, there was a significant difference in the allelic distribution of eNOS-CA between the two ethnic groups. The linkage results support the possibility that a susceptibility locus for pre-eclampsia resides in the 7q36 region, however, there is no definitive evidence to support the notion that the eNOS gene itself is responsible for susceptibility to pre-eclampsia. PMID: 10647900, UI: 20112426

2: Clin Genet 1999 Oct;56(4):289-96

Association of pre-eclampsia with common coding sequence variations in the lipoprotein lipase gene.

Hubel CA, Roberts JM, Ferrell RE

Marked dyslipidemia may contribute to endothelial cell dysfunction in re-eclampsia. Carriers of N291S or D9N missense mutations in the lipoproteinlipase (LPL) gene exhibit reductions in LPL activity and are predisposed to yslipidemia and cardiovascular disease. I Caucasians, the D9N variant is in strong linkage disequilibrium with the - 93T --> G promoter variant. A fourth LPL variant, S447X, is often associated with a beneficial lipid profile. We asked if the N291S and the combination D9N/- 93T --> G variants are more prevalent, and if the S447X variant is less prevalent, in Caucasian women with pre-eclampsia as compared with normal pregnancies. DNA amplification was followed by an allele-specific oligonucleotide ligation assay. Allele frequencies were analyzed with a chi2 table and Yates' correction. The N291S variant was identified in 11.1% of pre-eclamptics as compared with 2.9% of pregnancy controls (p = 0.008). All carriers of D9N were also carriers of - 93T --> G. The D9N/ - 93T --> G combined variant was found in 7.1% of pre-eclamptics as compared with 1.4% of pregnancy controls (p = 0.02). No individuals were carriers of both N291S and D9N/ - 93T --> G. Thus, 18.2% of pre-eclamptics had either of these LPL mutations compared with 4.3% of pregnancy controls (and 4.4%

of population controls). The frequency of the S447X variant did not differ among groups. We conclude that carriers of N291S or combined D9N/ - 93T --> G mutations in the LPL gene are at substantially increased risk of pre-eclampsia. PMID: 10636447, UI: 20100402

3: Hum Reprod 1999 Dec;14(12):3112-5

Activated protein C resistance shows an association with pregnancy-induced hypertension. Horstkamp BS, Kiess H, Kramer J, Riess H, Henrich W, Dudenhausen JW. A common mutation in the factor V gene, the Leiden mutation, is the most frequent genetic cause of resistance to activated protein C (APC). Recent studies have shown that the prevalence of APC resistance is associated with severe pregnancy-induced hypertension (PIH). Our objective was to determine whether the factor V Leiden mutation is more prevalent in patients who developed severe PIH than in normotensive pregnant women. In 70 women with a history of severe PIH, of whom 15 had pre-eclampsia, we investigated common coagulation factors as well as APC resistance (factor V related). We found that seven of these 70 women showed low values for APC. Out of these, five were heterozygousand none was homozygous for factor V Leiden mutation. In a control group ofnormotensive pregnant women we found a 3.0% rate of APC resistance and a 3.0% rate of carriers of the Leiden mutation. These results indicate a significantly higher prevalence of both APC resistance and factor V Leiden mutation in women with severe PIH. Placental infarctions and micro-embolisms are considered to be one of the principle pathophysiological changes in severe PIH. Our results suggest that APC resistance is a risk factor for severe PIH, in addition to its well-known role in macrothrombo-embolism. PMID: 10601105, UI: 20069832

4: Br J Obstet Gynaecol 1999 Mar;106(3):244-51

Maternal and fetal angiotensinogen gene allele sharing in pre-eclampsia.Morgan L, Crawshaw S, Baker PN, Broughton Pipkin F, Kalsheker N OBJECTIVE: To compare the angiotensinogen genotypes in normotensive and pre-eclamptic pregnancies in maternal and fetal samples. DESIGN: Prospective observational study. SETTING: University Hospital, Queen's Medical Centre, Nottingham. POPULATION: Forty-three women with pre-eclampsia and 84 normotensive pregnant women. Fetal samples were available for genotyping from 96% of the pregnancies. METHODS: Maternal and fetal DNA was genotyped at angiotensinogen codon 235 and at a dinucleotide repeat polymorphism in the 3' flanking region of the gene. Angiotensinogen and renin concentrations were measured in maternal plasma by radioimmunoassay. RESULTS: In contrast to earlier studies, no association was demonstrated between the angiotensinogen 235 Thr variant and pre-eclampsia. Normotensive pregnant women homozygous for this variant had significantly lower plasma angiotensinogen concentrations (median 2.2 ng AI/mL; IQR 1.8-3.0) than women homozygous for the 235 Met allele (3.6 ng AI/mL; IQR 2.5-4.1; P = 0.04). In pre-eclamptic pregnancies, 79% (11/14) of mothers heterozygous for the dinucleotide repeat allele designated A9 transmitted this allele to the fetus, more frequently than would be expected by chance (P = 0.02). The A9 allele was associated with low plasma angiotensinogen concentrations (P = 0.001) and high renin concentrations (P = 0.02) in normotensive women. CONCLUSIONS: There is no evidence that the angiotensinogen 235 Thr allele is associated with pre-eclampsia in the Nottingham population. The angiotensinogen 235 Thr allele is associated with low

plasma angiotensinogen concentrations in normotensive pregnant women, in contrast to the high levels associated with this variant in non-pregnant women, suggesting that regulation of angiotensinogen expression in normal pregnancy may differ significantly from that in the non-pregnant state. There is preliminary evidence that maternal-fetal transmission of an angiotensinogen allele associated with low plasma angiotensinogen concentrations is associated with pre-eclampsia. Impaired generation of angiotensin II at the maternal-fetal interface may be a factor in the pathogenesis of pre-eclampsia.

PMID: 10426644, UI: 99353716

5: Hypertension 1999 Jun;33(6):1338-41

Factor V Leiden and thermolabile methylenetetrahydrofolate reductase gene variants in an East Anglian preeclampsia cohort.

O'Shaughnessy KM, Fu B, Ferraro F, Lewis I, Downing S, Morris NH

Preeclampsia is a heritable condition that develops as a result of widespread vascular endothelial dysfunction. The thrombotic tendency in this condition has suggested a number of candidate genes, and there have been recent reports of positive association with the Leiden variant of factor V and the thermolabile variant of methylenetetrahydrofolate reductase. We attempted to reproduce these results in a large cohort of well-characterized women with preeclampsia, recruited prospectively within the East Anglian region of the United Kingdom. Women in the preeclampsia cohort (n=283) were genotyped for both the Leiden variant (G1691A) of factor V and the thermolabile variant (C677T) of methylenetetrahydrofolate reductase. Genotype and allele frequencies were compared with those of 2 control groups, one consisting of women recruited prospectively (n=100) from the same maternity hospital as the subjects and another consisting of normotensive women (n=100) from East Anglia. No significant differences were detected. Specifically, the carrier rate for the Leiden variant was 5.3% in the preeclampsia group and 5. 5% in the combined control group. T677 homozygotes for methylenetetrahydrofolate reductase were 11% and 11.5% in the 2 groups, respectively. We conclude that there is no evidence of association of preeclampsia with either of these 2 polymorphisms in our study population.

PMID: 10373212, UI: 99301877

6: Am J Obstet Gynecol 1998 Dec;179(6 Pt 1):1539-44

Preeclampsia is associated with reduced serum levels of placenta growth factor.

Torry DS, Wang HS, Wang TH, Caudle MR, Torry RJ

OBJECTIVES: Adequate vascular development of the placental bed is essential for

normal pregnancy. We assessed serum levels of placenta growth factor, anangiogenic factor, throughout normal pregnancy and determined its associationwith preeclampsia. STUDY DESIGN: Serum samples were collected from (1) 308healthy pregnant women throughout normal gestation, (2) at delivery from 30 eachgestational age-matched patients with normal pregnancy and preeclampsia, and (3)maternal and cord blood samples from normal deliveries with and without labor (n= 37 each). Placenta growth factor levels were determined with anantigen-capture enzyme-linked immunosorbent assay. RESULTS: Maternal placentagrowth factor levels during normal pregnancy increased from the first trimesterto the late second trimester; they subsequently declined from 30 weeks'gestation to delivery. Significantly less maternal placenta growth factor (P<.0001) was found in pregnancies complicated by preeclampsia, and laborsignificantly lowered placenta growth factor levels in both maternal (P =.0189)and cord serum samples (P <.0001). CONCLUSION: Decreased levels of placentagrowth factor during preeclampsia could influence endothelial cell andtrophoblast function, thereby contributing to the pathogenesis of the disease. PMID: 9855593, UI: 99072777

7: J Med Genet 1998 Aug;35(8):632-6

Distortion of maternal-fetal angiotensin II type 1 receptor allele transmission in pre-eclampsia.

Morgan L, Crawshaw S, Baker PN, Brookfield JF, Broughton Pipkin F, Kalsheker N

OBJECTIVE: To investigate the fetal angiotensin II type 1 receptor genotype inpre-eclampsia. DESIGN: Case-control study. POPULATION: Forty-one maternal-fetal pairs from pre-eclamptic pregnancies and 80 maternal-fetal pairs from normotensive pregnancies. METHODS: Maternal and fetal DNA was genotyped at three diallelic polymorphisms, at nucleotides 573, 1062, and 1166, in the coding exon of the angiotensin II type 1 receptor gene, and at a dinucleotide repeat polymorphism in its 3' flanking region. RESULTS: Allele and genotype frequenciesat the four polymorphic regions investigated did not differ between pre-eclamptic and normotensive groups, in either fetal or maternal samples. Mothers heterozygous for the dinucleotide repeat allele designated A4 transmitted this allele to the fetus in 15 of 18 informative pre-eclampticpregnancies and in eight of 26 normotensive pregnancies. This was greater than the expected probability in pre-eclamptic pregnancies (p=0.04) and less than expected in normotensive pregnancies (p<0.005). The 573T variant, which is in partial linkage disequilibrium with the A4 allele, showed a similar distortion of maternal-fetal transmission. CONCLUSION: Angiotensin II type 1 receptor gene expression in the fetus may contribute to the aetiology of pre-eclampsia. It is unclear whether susceptibility is conferred by the fetal genotype acting alone, or by allele sharing by mother and fetus. Possible mechanisms for the effect of the angiotensin II type 1 receptor gene are suggested by the association of the 573T variant with low levels of surface receptor expression on platelets. If receptor expression is similarly genetically determined in the placenta, responsiveness to angiotensin II may be affected, with the potential to influence placentation or placental prostaglandin secretion.

PMID: 9719367, UI: 98383872

8: Clin Genet 1998 Jun;53(6):478-81

Detection of factor V Leiden mutation in severe pre-eclamptic Hungarian women.

Nagy B, Toth T, Rigo J Jr, Karadi I, Romics L, Papp Z

Pre-eclampsia is a pregnancy-related disorder that complicates approximately 5% of all pregnancies and is cited as the primary cause of worldwide maternal and fetal mortality. The factor V Leiden mutation has been implicated in the development of severe pre-eclampsia. In order to investigate this association, a sample of 198 Hungarian women was recruited and enrolled in one of the three groups based on reproductive and health status: those as classified as pregnant and healthy (n = 71), those diagnosed as pregnant and severe pre-eclamptic (n =69), and those found to be healthy and non-pregnant (n = 58). The presence of factor V Leiden mutation was determined by using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism analysis (RFLP). We identified three (5.2%) heterozygous among healthy non-pregnant participants, five (7.0%) heterozygous among healthy pregnant participants and 13 (18.8%) heterozygous among pre-eclamptic women ($p < 0.05$, 95% CI). Our result supports the previous observation that carriers of factor V Leiden mutation are at increased risk for developing severe pre-eclampsia.

PMID: 9712539, UI: 98376384

9: Obstet Gynecol 1998 May;91(5 Pt 2):812-4

Severe preeclampsia associated with coinheritance of factor V Leiden mutation and protein S deficiency.

Kahn SR

University, Montreal, Quebec, Canada. susank@epid.jgh.mcgill.ca

BACKGROUND: Inherited thrombophilic disorders are associated with an increased risk of venous thromboembolism during pregnancy. Preliminary research suggests that these disorders might also increase the risk for preeclampsia. CASE: A 29-year-old primigravida developed severe, early onset preeclampsia and postpartum deep venous thrombosis. Subsequent testing revealed coinheritance of the factor V Leiden mutation and protein S deficiency. Heparin prophylaxis was administered during two subsequent pregnancies without recurrence of either preeclampsia or venous thromboembolism. CONCLUSION: Our patient's inherited thrombophilia may have played a role in the development of preeclampsia, and anticoagulation during subsequent pregnancies may have prevented preeclampsia recurrence. An association between inherited thrombophilic disorders and preeclampsia is biologically plausible.

PMID: 9572171, UI: 98231881

10: Thromb Haemost 1997 Jun;77(6):1052-4

Factor V Leiden, C > T MTHFR polymorphism and genetic susceptibility to preeclampsia.

Grandone E, Margaglione M, Colaizzo D, Cappucci G, Paladini D, Martinelli P, Montanaro S, Pavone G, Di Minno G

We performed a case-controlled study to investigate whether the FV Leiden mutation and the C > T677 polymorphism of the 5, 10 methylene tetrahydrofolate reductase (MTHFR) are associated with the occurrence of preeclampsia in 96 otherwise healthy preeclamptic women and 129 parous women as controls. FV Leiden carriers were 10 (10.5%) in cases and 3 (2.3%) in controls (OR: 4.9, 95% CI: 1.3-18.3). MTHFR TT homozygotes were 28 (29.8%) in cases and 24 (18.6%) in the control group (OR: 1.8, 95% CI 1.0-3.5). No difference in any of the polymorphisms was found between proteinuric (n = 45) and non-proteinuric (n =51) patients. Moreover, MTHFR polymorphism does not affect the association between FV Leiden and preeclampsia. In conclusion, FV Leiden mutation and MTHFR TT genotype are associated with the occurrence of preeclampsia, suggesting that, during pregnancy, women carrying these gene variants are prone to develop such a complication.

PMID: 9241730, UI: 97385716

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 02 November 1999 (02.11.99)	
International application No. PCT/IE99/00012	**Applicant's or agent's file reference** PL977PCT/MCG/TC
International filing date (day/month/year) 25 February 1999 (25.02.99)	**Priority date** (day/month/year) 25 February 1998 (25.02.98)
Applicant O'BRIEN, Margaret et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

06 September 1999 (06.09.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Juan Cruz
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

Gates, M.C.E.
TOMKINS & CO.
5 Dartmouth Road
Dublin 6
IRLANDE

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	19.11.1999
Applicant's or agent's file reference: PL977PCT/MCG/TC	**REPLY DUE** **within 3 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IE99/00012	25/02/1999	25/02/1998

International Patent Classification (IPC) or both national classification and IPC

C12Q1/68

Applicant

NATIONAL UNIVERSITY OF IRELAND, CORK et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☒	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☒	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 25/06/2000.

Name and mailing address of the international preliminary examining authority:

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer / Examiner

Weaver, M

Formalities officer (incl. extension of time limits)

Danti, B
Telephone No. +49 89 2399 2735

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".)*:

Description, pages:

1-56 as originally filed

Claims, No.:

1-26 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application,

☒ claims Nos. 16 and 17 (part), 19-22, 24-26 ,

because:

☒ the said international application, or the said claims Nos. 16 and 17 (part), 20-22, 24-26 in respect of industrial applicability relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sh et

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☒ the claims, or said claims Nos. 19-22, 25, 26 are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	
Inventive step (IS)	Claims	1-18, 23, 24
Industrial applicability (IA)	Claims	

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Section I

The sequence listing pages 1/9-9/9 are also included in the basis of this opinion.

Section III

1. Claims 16 and 17, insofar as they also encompass diagnostics methods performed on the human body, 20 - 22 and 24 - 26 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

2. Claims 19 - 22, 25 and 26 are directed to methods for screening potential therapeutic agents for treatment of e.g. PE and IUGR, methods of preventing e.g PE and IUGR, methods for improving pregnancy success, methods for induction of tolerance and methods for the treatment of autoimmune disease, none of which enjoy any substantial support in terms of concrete examples in the application as filed. There are no concrete indications of what may constitue a potential therapeutic agent, nor of whether any such purported agents actually have any therapeutic effect on any of the clinical conditions (e.g. PE or IUGR) listed in claims 19 - 22. Moreover, there is no evidence whatsoever in the application as filed of any tolerance induction or successful treatment of autoimmune disease by administering any of the products referred to in claims 25 and 26. The passages on pages 39 - 42 of the description represent mere speculation on potential consequences of the knowledge that differences in HLA-G expression are associated with a number of abnormal pregnancy states, in particular PE and IUGR (see Section V). These passages in no way represent a clear and unambiguous disclosure that the suggested uses and methods actually yield the desired result.

   Consequently, these claims are so inadequately supported by the description that no meaningful opinion can be formed the novelty, inventive step and industrial applicability of the subject-matter of these claims (Article 34(4)(a)(ii) PCT).

Section V

1. Despite the lack of clarity of claim 1 (see Section VIII), it is possible to establish that its subject-matter is directed to a method for the diagnosis of e.g. pre-eclampsia (PE), eclampsia or intrauterine growth retardation (IUGR) by determining the presence or absence, in a body fluid or tissue sample, of an HLA-G associated parameter, such as an encoding nucleic acid, the transcribed mRNA, the protein itself or the effect of HLA-G on cells or molecules.

2. D1 (Journal of Reproductive Immunology, vol. 32, no. 2, December 1996, page 111-23; in particular the abstract; page 114 "3. Results"; Table 1; page 120, 2nd and 3rd paragraphs) describes low levels of HLA-G exon 2 and 3 polymorphism in an inbred population, which shows no deleterious effects on the pregnancy outcome. Moreover, the population has a much lower incidence of recurrent miscarriage, preterm labour, IUGR, PE or other pregnancy complications. A total of seven polymorphisms were studied: three each in exons 2 and 3 (including the codon 93 C/T allele) and the deletion polymorphism in exon 8.

3. D2 (Journal of Reproductive Immunology, vol. 36, no. 1-2, November 1997, page 1-21; in particular page 15, point 4.2) discloses reduced expression of HLA-G1 protein in term placenta from PE pregnancies and suggests that decreased HLA-G protein expression, whether by mutation or polymorphism in the HLA-G gene, may lead to fetal loss, IUGR or PE. Similar conclusions of an association between reduced HLA-G expression and PE are to be found in D3 (Current Problems in Obstetrics, Gynecology and Fertility, vol. 21, no. 1, January 1998, pages 6-23; page 12, lhc, line 37 - rhc, line 12), D4 (Journal of the American Society of Nephrology, vol. 9, September 1997, page 330A) and D5 (American Journal of Reproductive Immunology, vol. 36, no. 6, December 1996, pages 349-58; see e.g. the abstract). In addition, D6 (American Journal of Obstetrics and Gynecology, vol. 170, no. 1(part 2) , 1994, page 289) discloses an association between PE and the placental expression of a variant allele of HLA-G.

4. The clear and unambiguous teaching of all of these documents is that differences in HLA-G expression are associated with a number of abnormal pregnancy states, in particular PE and IUGR. It would then be a straightforward matter, devoid of

any inventive merit, for the skilled person to apply the collective teaching of these documents to solve the problem of providing a means of determining susceptibility to any of the abnormal pregnancy conditions, such as PE, mentioned in D1 - D6. He would also know from his general biochemical and molecular biological knowledge that differences in HLA-G expression could be determined in a number of ways, such as assaying nucleic acids, the protein itself or the effect the protein has on other systems.

Claim 1 thus lacks an inventive step and does not meet the requirements of Article 33(3) PCT.

5. Dependent claims 2 - 15 do not appear contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of inventive step, given the disclosures of D1 - D6 (Article 33(3) PCT).

6. The above arguments (points 1 - 5) apply equally to claim 23, directed to a kit suitable for use in the methods of clams 1 - 15. Claim 23 thus also lacks an inventive step (Article 33(3) PCT).

7. Although claim 16 lacks clarity (see Section VIII), it is possible to establish that its subject-matter is directed, at least in part, to a method of comparison of (i) the expression level of any gene or any protein in cells which have interacted with HLA-G expressing cells (presumably in normal subjects, although this is not stated in the claim) with (ii) the same expression level under the same conditions in patients with e.g. PE or IUGR, in order to identify possible candidates for diagnostic indicators or drug targets for use in patients having one of these clinical conditions.

However, given the known association of differences in HLA-G expression with a number of abnormal pregnancy states, in particular PE and IUGR, it would be obvious for the skilled person, in the light of the disclosures of D1 - D6, to carry out such screening methods when seeking to provide a method of identifying e.g. possible diagnostic markers of e.g. PE or IUGR.

Claim 16 thus lacks an inventive step and does not meet the requirements of Article 33(3) PCT.

8. Dependent claim 17 relates to standard methods of measurement of gene or protein expression and cannot contribute anything inventive to the subject-matter of claim 16 (Article 33(3) PCT).

9. Although claim 18 is unclear (see Section VIII), at least one of the embodiments is a composition which contains a pharmaceutically effective amount of HLA-G only.

As HLA-G is known as such (see any of D1 - D6), it would be an obvious matter for te skilled person to formulate an amount of HLA-G in a composition which would be appropriate for the intended pharmaceutical application.

Claim 18 thus lacks an inventive step (Article 33(3) PCT).

10. The entire gene sequence of HLA-G is acknowledged to be known as are polymorphisms of exons 2, 3 and 8 (see page 3, lines 21 - 29 of the present application and D1). As at least some of the nucleic acids sequences referred to in claim 24 are designed to and are capable of determining whether these polymorphisms are present (see page 21, line 29 - page 22, line 24 of the present application) and given the known association of these polymorphisms with recurrent miscarriage, preterm labour, IUGR, PE and other pregnancy complications (see point 2 above) it would be an obvious expedient, devoid of any inventive merit, for the skilled person to use nucleic acid sequences which are able to detect such polymorphisms when seeking to provide a means of assessing potential susceptibility to such clinical conditions.

Claim 24 thus lacks an inventive step and does not meet the requirements of Article 33(3) PCT.

11. For the assessment of the present claims 16 and 17, insofar as they also encompass diagnostics methods performed on the human body, 20 - 22 and 24 - 26 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the

formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Section VII

Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1 - D6 is not mentioned in the description, nor are these documents identified therein.

Section VIII

1. The following embodiments of claims 1, 4 and 11 are not supported by any concrete evidence in the form of examples which would allow the skilled person to reproduce the alleged invention as claimed:

   (i) "HLA-G linked nucleic acid [sequence]" (e.g. claim 1, part(b));

   (ii) "proteins encoded by HLA-G linked genes" (e.g. claim 1, part(c));

   (iii) "mRNA transcribed from HLA-G linked genes" (e.g. claim 1, part(e)).

   This subject-matter thus does not meet the requirements of Article 5 PCT.

2. The excessive use of the terms "and/or" and "or" in the claims renders them unclear, as it is difficult, if not impossible, to determine the entire scope of the claim given the extremely high number of potential embodiments generated by the use of these terms, in particular "and/or". As an example, all of the occurrences of these terms in claim 1, when taken together, result in over 30000 possible embodiments. This difficulty in determining the matter for which protection is sought also places an undue burden on others seeking to establish the extent of the protection.

Hence, the claims as a whole do not meet the requirements of Article 6 PCT.

3. Claim 19 does not meet the requirements of Article 6 PCT as, although directed to a method, the different embodiments (a) - (j) merely state what is desired to be done without indicating, in terms of technical method features, how this is to be achieved (see also Rule 6.3(a) PCT).

4. The term "an effective HLA-G" used in part (a) of claim 20 is unclear in context (Article 6 PCT).

inhibitors of cytokines and/or tumour necrosis factor alpha and/or derivatives of cytokines and/or tumour necrosis factor-alpha, optionally with pharmaceutically-acceptable carriers or excipients.

19. A method for screening potential pre-eclampsia and eclampsia and intrauterine growth retardation and miscarriage and miscarriage-related infertility therapeutic agents selected from:

a) identifying agents which alter the expression of HLA-G;

b) identifying agents which alter the activity of HLA-G;

c) identifying agents which mimic the action of HLA-G;

d) identifying agents which bind to HLA-G;

e) identifying peptides which bind to HLA-G;

f) identifying agents which bind to HLA-G receptors;

g) identifying expressed genes using DNA probe arrays in a cellular background in HLA-G expressing cells and / or blood mononuclear cells interacting with HLA-G and / or cells expressing HLA-G interacting with blood mononuclear cells;

h) identifying expressed proteins using mass spectrometry methods in HLA-G expressing cells and / or blood mononuclear cells interacting with HLA-G and / or cells expressing HLA-G interacting with blood mononuclear cells;

i) identifying expressed proteins using mass spectrometry methods in HLA-G expressing cells and / or blood mononuclear cells interacting with HLA-G and / or cells expressing HLA-G interacting with blood mononuclear cells;

j) screening sperm and/or semen and/or female reproductive tissue for agents:

i) which alter the expression of HLA-G in fertilised eggs and/or embryos;

ii) which alter the cell cleavage rate of fertilised eggs and/or embryos;

iii) which induce cellular factors in cells in culture and/or cells *in vivo* that alter the cell cleavage rate of fertilised eggs and/or embryos.

20. A method for the prevention of pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility selected from:

a) treatment of a female with all or part of a pharmaceutically effective amount of an effective HLA-G and /or peptides which bind to HLA-G and / or cells expressing HLA-G;

b) treatment of a female with all or part of a pharmaceutically effective amount of molecules or inhibitors of molecules whose level or activity is directly or indirectly altered by HLA-G action;

c) treatment of a female with all or part of a pharmaceutically effective amount of molecules which inhibit the interaction between HLA-G and one or more of its receptors;

d) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters HLA-G expression:

e) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters HLA-G related blood mononuclear cell activity;

f) treatment of a female with all or part of a pharmaceutically effective amount of an agent which mimics all or part of HLA-G action;

g) treatment of a female with blood mononuclear cells that recognise foetal and / or self HLA-G;

h) treatment of a female with HLA-G and / or cells expressing HLA-G or variants thereof;

i) treatment of a female with one or more antibodies which bind to HLA-G and / or cells expressing HLA-G and / or any receptor for HLA-G;

j) introduction of one or more variants of the HLA-G gene and /or its receptor into a female and / or male;

k) introduction of an inhibitor of expression of the HLA-G gene and/or its receptor into a female and/or male;

l) inactivation of one or more variants of the HLA-G gene and/or its receptor in a female and/or male.

21. A method for improving pregnancy success selected from:

a) pre-treating the female with sperm and/or attenuated forms thereof, and/or semen and/or fractions thereof from a male with a known HLA-G genotype, prior to mating with a male of a different HLA-G genotype, and/or in vitro fertilisation using sperm from a male of a different HLA-G genotype and/or embryo transfer where the male HLA-G is of a different HLA-G genotype;

b) mixing sperm of a known HLA-G genotype with sperm and/or attenuated forms thereof, and/or semen and/or fractions thereof from a male with a different HLA-G genotype prior to in vitro fertilisation.

22. A method as claimed in claim 21 wherein fertility and / or pregnancy outcome are improved by selection of male and / or female partners and / or sperm and / or ova and / or recipients of fertilised eggs and / or zygotes / and / or embryos so that (a) their HLA-G and /or HLA genotypes and /or serotypes or (b) the activity of their HLA-G and / or blood mononuclear cells interacting with HLA-G are indicative of normal pregnancy outcomes and / or not associated with pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility.

23. A test kit for the diagnosis of susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility or for monitoring progress of pregnancy comprising:

a) oligonucleotide primers for amplification of all or part of the HLA-G gene and /or HLA-G linked DNA;

b) amplification reagents for amplification of genomic DNA and / or RNA segments, selected from a DNA / RNA polymerase, a reverse transcriptase, the deoxyribonucleotides dATP, dCTP, dGTP, dTTP and dUTP. and /or ribonucleotides ATP, CTP, GTP, TTP and UTP, and reaction buffer;

c) reagents for identifying sequence variants in DNA and / or RNA;

d) control DNA and /or RNA.

24. Use of a DNA sequence selected from any one of Sequence I.D.s 1 to 21 for diagnosis of susceptibility to or in a test kit for the diagnosis of susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility, for monitoring progress of pregnancy, for use in the manufacture of a medicament, in a method for screening potential therapeutic agents, in a method for screening for potential diagnostic indicators and/or drug targets, in a method for improving pregnancy success or in a method for the prevention of pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility, for monitoring progress of pregnancy.

25. A method for induction of tolerance in a host to a non-self tissue which comprises administering HLA-G and /or HLA-G loaded with peptides from the non-self tissue and /or HLA-G expressing cells derived from or related to the non-self tissue, and/or a non-self tissue bearing an introduced HLA-G so that HLA-G is expressed in all or part of the tissue.

26. A method for the treatment of autoimmune disease which comprises administering HLA-G and /or HLA-G loaded with peptides from a self and/or non-self tissue and / or with specific autoimmune antigen and /or HLA-G expressing cells from a self and/or non-self tissue and/or a self and/or self tissue bearing an introduced HLA-G gene so that HLA-G is expressed in all or part of the tissue.

genotype, and/or in vitro fertilisation using sperm from a male of a different HLA-G genotype and/or embryo transfer where the male HLA-G is of a different HLA-G genotype;

b) mixing sperm of a known HLA-G genotype with sperm and/or attenuated forms thereof, and/or semen and/or fractions thereof from a male with a different HLA-G genotype prior to in vitro fertilisation.

22. A method as claimed in claim 21 wherein fertility and / or pregnancy outcome are improved by selection of male and / or female partners and / or sperm and / or ova and / or recipients of fertilised eggs and / or zygotes / and / or embryos so that (a) their HLA-G and /or HLA genotypes and /or serotypes or (b) the activity of their HLA-G and / or blood mononuclear cells interacting with HLA-G are indicative of normal pregnancy outcomes and / or not associated with pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility.

23. A test kit for the diagnosis of susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility or for monitoring progress of pregnancy comprising:

a) oligonucleotide primers for amplification of all or part of the HLA-G gene and /or HLA-G linked DNA;

b) amplification reagents for amplification of genomic DNA and / or RNA segments, selected from a DNA / RNA polymerase, a reverse transcriptase, the deoxyribonucleotides dATP, dCTP, dGTP, dTTP and dUTP, and /or ribonucleotides ATP, CTP, GTP, TTP and UTP, and reaction buffer;

c) reagents for identifying sequence variants in DNA and /or RNA;

d) control DNA and /or RNA.

24. Use of a DNA sequence selected from any one of Sequence I.D.s 1 to 21 for diagnosis of susceptibility to or in a test kit for the diagnosis of susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility, for monitoring progress of pregnancy, for use in the manufacture of a medicament, in a method for screening potential therapeutic agents, in a method for screening for potential diagnostic indicators and/or drug targets, in a method for improving pregnancy success or in a method for the prevention of pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility, for monitoring progress of pregnancy.

25. A method for induction of tolerance in a host to a non-self tissue which comprises administering HLA-G and /or HLA-G loaded with peptides from the non-self tissue and /or HLA-G expressing cells derived from or related to the non-self tissue, and/or a non-self tissue bearing an introduced HLA-G so that HLA-G is expressed in all or part of the tissue.

26. A method for the treatment of autoimmune disease which comprises administering HLA-G and /or HLA-G loaded with peptides from a self and/or non-self tissue and / or with specific autoimmune antigen and /or HLA-G expressing cells from a self and/or non-self tissue and/or a self and/or self tissue bearing an introduced HLA-G gene so that HLA-G is expressed in all or part of the tissue.

add β')

inhibitors of cytokines and/or tumour necrosis factor alpha and/or derivatives of cytokines and/or tumour necrosis factor-alpha, optionally with pharmaceutically-acceptable carriers or excipients.

19. Use of HLA-G or HLA-G expressing genes in a method of screening for potential therapeutic agents for the treatment of a condition selected from:-. pre-eclampsia, eclampsia, intrauterine growth retardation, susceptibility to miscarriage and miscarriage-related infertility.

20. A method for the prevention of pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility selected from:

a) treatment of a female with all or part of a pharmaceutically effective amount of a HLA-G and /or peptides which bind to HLA-G and / or cells expressing HLA-G;

b) treatment of a female with all or part of a pharmaceutically effective amount of molecules or inhibitors of molecules whose level or activity is directly or indirectly altered by HLA-G action;

c) treatment of a female with all or part of a pharmaceutically effective amount of molecules which inhibit the interaction between HLA-G and one or more of its receptors;

d) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters HLA-G expression;

e) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters HLA-G related blood mononuclear cell activity;

f) treatment of a female with all or part of a pharmaceutically effective amount of an agent which mimics all or part of HLA-G action;

g) treatment of a female with blood mononuclear cells that recognise foetal and / or self HLA-G;

h) treatment of a female with HLA-G and / or cells expressing HLA-G or variants thereof;

i) treatment of a female with one or more antibodies which bind to HLA-G and / or cells expressing HLA-G and / or any receptor for HLA-G;

j) introduction of one or more variants of the HLA-G gene and /or its receptor into a female and / or male;

k) introduction of an inhibitor of expression of the HLA-G gene and/or its receptor into a female and/or male;

l) inactivation of one or more variants of the HLA-G gene and/or its receptor in a female and/or male.

21. A method for improving pregnancy success selected from:

a) pre-treating the female with sperm and/or attenuated forms thereof, and/or semen and/or fractions thereof from a male with a known HLA-G genotype, prior to mating with a male of a different HLA-G

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

Gates, M.C.E.
TOMKINS & CO.
5 Dartmouth Road
Dublin 6
IRLANDE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

	Date of mailing, *(day/month/year)* 26.05.2000
Applicant's or agent's file reference PL977PCT/MCG/TC	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IE99/00012	25/02/1999	25/02/1998

Applicant
NATIONAL UNIVERSITY OF IRELAND, CORK et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Borinski, W Tel.+49 89 2399-8237

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
PL977PCT/MCG/TC		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IE 99/00012	25/02/1999	25/02/1998
Applicant		
NATIONAL UNIVERSITY OF IRELAND, CORK et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [X] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. –

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IE 99/00012

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 20, 21, 25 and 26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: partially 25 and 26
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: partially 25 and 26.

Method claims 25 and 26 (also see Remark) have been searched only insofar as the methods relate to susceptibility to normal pregnancy, pre-eclampsia and/ or eclampsia and/ or intrauterine growth retardation and/ or susceptibility to miscarriage and/ or miscarriage-related infertility.

INTERNATIONAL SEARCH REPORT

International Application No
T/IE 99/00012

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12Q1/02 C12N15/12 G01N33/50 G01N33/564
A61K38/17 A61K35/14 A61K39/395 //C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	OBER C ET AL: "Population genetic studies of HLA-G: allele frequencies and linkage disequilibrium with HLA-A " JOURNAL OF REPRODUCTIVE IMMUNOLOGY, vol. 32, no. 2, December 1996, page 111-23 XP002105897 see page 120, paragraph 2 - paragraph 3 ---	1-24
A	OBER C ET AL: "HLA-G polymorphisms: neutral evolution or novel function" JOURNAL OF REPRODUCTIVE IMMUNOLOGY, vol. 36, no. 1-2, November 1997, page 1-21 XP002105898 see the whole document --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 June 1999	06/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	HUMPHREY K ET AL: "HLA-G deletion polymorphism and pre-eclampsia/eclampsia" BRITISH JOURNAL OF OBSTETRICS AND GYNAECOLOGY, vol. 102, no. 9, September 1995, pages 707-10, XP002105899 see the whole document ---	1-24
A	MCMASTER M ET AL: "Immunology of human pregnancy" CURRENT PROBLEMS IN OBSTETRICS, GYNECOLOGY AND FERTILITY, vol. 21, no. 1, January 1998, pages 6-23, XP002105900 see page 9 - page 13 ---	1-24
A	KARHUKORPI J ET AL: "HLA-G polymorphism in Finnish couples with recurrent spontaneous miscarriage" BRITISH JOURNAL OF OBSTETRICS AND GYNAECOLOGY, vol. 104, no. 10, October 1997, pages 1212-14, XP002105901 see the whole document ---	1-24
A	STEFFENSEN R ET AL: "HLA-G PCR-RPFL typing of woman with multiple spontaneous abortions" HUMAN BIOLOGY, vol. 47, no. 1-2, 1996, page 147 XP002105902 see abstract P798 ---	1-24
A	HENNESSY A ET AL: "Reduced expression of immunosupressor genes in preeclampsia (PE)" JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY, vol. 9, September 1997, page 330A XP002105903 see abstract A1376. ---	1-24
A	HARA N ET AL: "Altered expression of human leukocyte antigen G (HLA-G) on extravillous trophoblasts in preeclampsia" AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY, vol. 36, no. 6, December 1996, pages 349-58, XP002105904 see abstract on 349 --- -/--	1-24

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INTERNATIONAL SEARCH REPORT

International Application No
T/IE 99/00012

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MAIN E ET AL: "Nulliparous preeclampsia (PE) is associated with placental expression of a variant allele of the new histocompatibility gene: HLA-G" AMERICAN JOURNAL OF OBSTETRICS AND GYNECOLOGY, vol. 170, no. 1(part 2) , 1994, page 289 XP002105905 see the whole document	1-26
A	CAROSELLA E D ET AL: "HLA-G revisited" IMMUNOLOGY TODAY, vol. 17, no. 9, 1 September 1996, page 407-409 XP004034736 see the whole document	1-24
A	WO 96 31604 A (UNIV CALIFORNIA) 10 October 1996 see the whole document	25,26
A	WO 95 31472 A (UNIV TEXAS) 23 November 1995	25,26
A	FR 2 717 498 A (COMMISSARIAT ENERGIE ATOMIQUE) 22 September 1995 see the whole document	25,26

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INTERNATIONAL SEARCH REPORT

[redacted]ation on patent family members

International Application No
[redacted]T/IE 99/00012

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9631604 A	10-10-1996	AU 696118 B	03-09-1998
		AU 5256896 A	23-10-1996
		CA 2213620 A	10-10-1996
		EP 0819171 A	21-01-1998
		JP 11503320 T	26-03-1999
WO 9531472 A	23-11-1995	AU 688914 B	19-03-1998
		AU 2513395 A	05-12-1995
		CA 2190576 A	23-11-1995
		EP 0759930 A	05-03-1997
		JP 10501690 T	17-02-1998
FR 2717498 A	22-09-1995	EP 0677582 A	18-10-1995
		US 5856442 A	05-01-1999

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PL977PCT/MCG/TC		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IE99/00012	25/02/1999	25/02/1998
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant NATIONAL UNIVERSITY OF IRELAND, CORK et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
06/09/1999	26.05.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Weaver, M Telephone No. +49 89 2399 8689

Form PCT/IPEA/409 (cover sheet) (January 1994)

concrete evidence in the form of examples which would allow the skilled person to reproduce the alleged invention as claimed:

(i) "HLA-G linked nucleic acid [sequence]" (e.g. claim 1, part(b));

(ii) "proteins encoded by HLA-G linked genes" (e.g. claim 1, part(c));

(iii) "mRNA transcribed from HLA-G linked genes" (e.g. claim 1, part(e)).

This subject-matter thus does not meet the requirements of Article 5 PCT.

2. The excessive use of the terms "and/or" and "or" in the claims renders them unclear, as it is difficult, if not impossible, to determine the entire scope of the claim given the extremely high number of potential embodiments generated by the use of these terms, in particular "and/or". As an example, all of the occurrences of these terms in claim 1, when taken together, result in over 30000 possible embodiments. This difficulty in determining the matter for which protection is sought also places an undue burden on others seeking to establish the extent of the protection.

Hence, the claims as a whole do not meet the requirements of Article 6 PCT.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IE99/00012

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-56	as originally filed				

Claims, No.:

1-17,18 (part)	as originally filed				
18 (part),19-26	as received on	21/02/2000	with letter of	16/02/2000	

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 16 and 17 (part), 19-22, 24-26 .

because:

☒ the said international application, or the said claims Nos. 16 and 17 (part), 20-22, 24-26 in respect of

industrial applicability relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☒ the claims, or said claims Nos. 19-22, 25, 26 are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-18, 23, 24
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-15, 23, 24
	No:	Claims	16-18
Industrial applicability (IA)	Yes:	Claims	1-15, 18, 23 and 16, 17 (partly)
	No:	Claims	

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

While the applicant's observations submitted with the amended claims have been considered, the previously expressed opinion is nevertheless maintained in part for the reasons indicated below.

Section I

The sequence listing pages 1/9-9/9 are also included in the basis of this opinion.

Section III

1. Claims 16, 17 and 19, insofar as they also encompass diagnostics methods performed on the human body, 20 - 22 and 24 - 26 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

2. Claims 19 - 22, 25 and 26 are directed to methods for screening potential therapeutic agents for treatment of e.g. PE and IUGR, methods of preventing e.g PE and IUGR, methods for improving pregnancy success, methods for induction of tolerance and methods for the treatment of autoimmune disease, none of which enjoy any substantial support in terms of concrete examples in the application as filed. There are no concrete indications of what may constitue a potential therapeutic agent, nor of whether any such purported agents actually have any therapeutic effect on any of the clinical conditions (e.g. PE or IUGR) listed in claims 20 - 22. Moreover, there is no evidence whatsoever in the application as filed of any tolerance induction or successful treatment of autoimmune disease by administering any of the products referred to in claims 25 and 26. The passages on pages 39 - 42 of the description represent mere speculation on potential consequences of the knowledge that there is linkage between differences in HLA-G expression and a number of abnormal pregnancy states, in particular PE and IUGR (see Section V). These passages in no way represent a clear and unambiguous disclosure that the suggested uses and methods actually yield the desired result.

   Consequently, these claims are so inadequately supported by the description that

no meaningful opinion can be formed on the novelty, inventive step and industrial applicability of the subject-matter of these claims (Article 34(4)(a)(ii) PCT).

Section V

1. Despite the lack of clarity of claim 1 (see Section VIII), it is possible to establish that its subject-matter is directed to a method for the diagnosis of e.g. pre-eclampsia (PE), eclampsia or intrauterine growth retardation (IUGR) by determining the presence or absence, in a body fluid or tissue sample, of an HLA-G associated parameter, such as an encoding nucleic acid, the transcribed mRNA, the protein itself or the effect of HLA-G on cells or molecules.

2. D1 (Journal of Reproductive Immunology, vol. 32, no. 2, December 1996, page 111-23; in particular the abstract; page 114 "3. Results"; Table 1; page 120, 2nd and 3rd paragraphs) describes low levels of HLA-G exon 2 and 3 polymorphism in an inbred population, which shows no deleterious effects on the pregnancy outcome. Moreover, the population has a much lower incidence of recurrent miscarriage, preterm labour, IUGR, PE or other pregnancy complications. A total of seven polymorphisms were studied: three each in exons 2 and 3 (including the codon 93 C/T allele) and the deletion polymorphism in exon 8.

3. D2 (Journal of Reproductive Immunology, vol. 36, no. 1-2, November 1997, page 1-21; in particular page 15, point 4.2) discloses reduced expression of HLA-G1 protein in term placenta from PE pregnancies and suggests that decreased HLA-G protein expression, whether by mutation or polymorphism in the HLA-G gene, may lead to fetal loss, IUGR or PE. Similar conclusions of an association between reduced HLA-G expression and PE are to be found in D3 (Current Problems in Obstetrics, Gynecology and Fertility, vol. 21, no. 1, January 1998, pages 6-23; page 12, lhc, line 37 - rhc, line 12), D4 (Journal of the American Society of Nephrology, vol. 9, September 1997, page 330A) and D5 (American Journal of Reproductive Immunology, vol. 36, no. 6, December 1996, pages 349-58; see e.g. the abstract). In addition, D6 (American Journal of Obstetrics and Gynecology, vol. 170, no. 1(part 2) , 1994, page 289) discloses an association between PE and the placental expression of a variant allele of HLA-G.

4. Although these documents teach that differences in HLA-G expression are associated with a number of abnormal pregnancy states, in particular PE and IUGR, there is no disclosure whatsoever of linkage between the two, i.e. that differences in HLA-G expression play a direct role in pregnancy outcome and can thus be used in the determination of susceptibility to any of the abnormal pregnancy conditions, such as PE, mentioned in D1 - D6.

   Claim 1 is thus novel and inventive step and meets the requirements of Article 33(2) and (3) PCT.

5. Dependent claims 2 - 15, together with claim 23, directed to a kit suitable for use in the methods of clams 1 - 15, and use claim 24 can also be acknowledged as novel and inventive in combination with claim 1 for similar reasons (Article 33(2) and (3) PCT).

6. Although claim 16 lacks clarity (see Section VIII), it is possible to establish that its subject-matter is directed, at least in part, to a method of comparison of (i) the expression level of any gene or any protein in cells which have interacted with HLA-G expressing cells (presumably in normal subjects, although this is not stated in the claim) with (ii) the same expression level under the same conditions in patients with e.g. PE or IUGR, in order to identify possible candidates for diagnostic indicators or drug targets for use in patients having one of these clinical conditions.

   However, given the known association of differences in HLA-G expression with a number of abnormal pregnancy states, in particular PE and IUGR, it would be obvious for the skilled person, in the light of the disclosures of D1 - D6, to carry out such screening methods when seeking to provide a method of identifying e.g. possible diagnostic markers of e.g. PE or IUGR.

   It should be noted in this context that is irrelevant whether the connection between differences in HLA-G expression and a number of abnormal pregnancy states, in particular PE and IUGR, is one of association or linkage. In both cases differences in HLA-G expression would exist and thus be useable in the said screening methods.

Claim 16 thus lacks an inventive step and does not meet the requirements of Article 33(3) PCT.

7. Dependent claim 17 relates to standard methods of measurement of gene or protein expression and cannot contribute anything inventive to the subject-matter of claim 16 (Article 33(3) PCT).

8. Although claim 18 is unclear (see Section VIII), at least one of the embodiments is a composition which contains a pharmaceutically effective amount of HLA-G only.

   As HLA-G is known as such (see any of D1 - D6), it would be an obvious matter for the skilled person to formulate an amount of HLA-G in a composition which would be appropriate for the intended pharmaceutical application.

   Claim 18 thus lacks an inventive step (Article 33(3) PCT).

9. For the assessment of the present claims 16, 17 and 19, insofar as they also encompass diagnostics methods performed on the human body, 20 - 22 and 24 - 26 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Section VII

Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1 - D6 is not mentioned in the description, nor are these documents identified therein.

Section VIII

1. The following embodiments of claims 1, 4 and 11 are not supported by any

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12Q 1/68, 1/02, C12N 15/12, G01N 33/50, 33/564, A61K 38/17, 35/14, 39/395 // C12N 5/10

A1

(11) International Publication Number: WO 99/43851

(43) International Publication Date: 2 September 1999 (02.09.99)

(21) International Application Number: PCT/IE99/00012

(22) International Filing Date: 25 February 1999 (25.02.99)

(30) Priority Data:

980134	25 February 1998 (25.02.98)	IE
980668	12 August 1998 (12.08.98)	IE

(71) Applicant *(for all designated States except US)*: NATIONAL UNIVERSITY OF IRELAND, CORK [IE/IE]; College Road, Cork (IE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: O'BRIEN, Margaret [IE/IE]; Flat 2, 63 Shandon Street, Cork (IE). BERMINGHAM, John [IE/IE]; 37 Castleknock Crescent, Castleknock, County Dublin (IE). QUANE, Kathleen, A. [IE/IE]; Monanimy Upper, Killavullen, County Cork (IE). JENKINS, David, M. [GB/IE]; 27 The Highlands, Passage West, Cork (IE). McCARTHY, Tommie, V. [IE/IE]; Vista Villa, Montenotte, Cork (IE).

(74) Agents: GATES, Marie, Christina, Esther et al.; Tomkins & Co., 5 Dartmouth Road, Dublin 6 (IE).

(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HLA LINKED PRE-ECLAMPSIA AND MISCARRIAGE SUSCEPTIBILITY GENE

(57) Abstract

The invention relates to the identification of a susceptibility gene for pre-eclampsia and eclampsia and provides methods and diagnostic kits for diagnosing susceptibility to normal pregnancy, pre-eclampsia, eclampsia, intrauterine growth retardation, miscarriage or miscarriage-related infertility. The invention is based on analysing HLA-G or HLA-G linked nucleic acid, or HLA-G protein or HLA-G mRNA or cells or molecules whose concentration changes as a result of HLA-G action. The invention also provides pharmaceutical compositions for and methods of treatment of the above conditions.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
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CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
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CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
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HLA linked pre-eclampsia and miscarriage susceptibility gene

The present invention relates to a susceptibility gene for pre-eclampsia and eclampsia, and the use of such a gene in methods for diagnosing susceptibility to these diseases. The invention also relates to a test kit for diagnosis of susceptibility and to pharmaceutical compositions for the prevention or treatment of the diseases. The invention can also be used in the diagnosis of susceptibility to miscarriage and/or miscarriage-related infertility and/or intrauterine growth retardation.

Specifically, the present invention relates to methods and materials used to detect a HLA linked human pre-eclampsia and miscarriage predisposing gene (HLA-G), some alleles of which, or linked alleles of linked genes of which, cause susceptibility to pre-eclampsia and miscarriage. More specifically, the invention relates to sequence variation in the HLA-G gene and linked genes and their use in the diagnosis of susceptibility to pre-eclampsia and miscarriage. The invention further relates to sequence variations in the HLA-G gene and their use in diagnosis and prognosis of pre-eclampsia and miscarriage. Additionally, the invention relates to the therapy for pre-eclampsia and miscarriage and for susceptibility to pre-eclampsia and miscarriage including protein therapy, gene therapy and protein mimetics. The invention also relates to screening for drugs for pre-eclampsia and miscarriage therapy and for susceptibility to pre-eclampsia and miscarriage therapy. Finally, the invention relates to the screening of the HLA-G gene and linked genes for sequence variations which are useful for diagnosing susceptibility to pre-eclampsia and miscarriage.

Pre-eclampsia is the major cause of foetal and maternal morbidity and mortality with probable long term adverse effects on health due to the prolonged associated intrauterine hypoxia. Pre-eclampsia occurs in approximately five to ten percent of all population births and is uniquely a disease of pregnancy. Acute pathological changes begin to resolve soon after delivery. The pathologic mechanisms causing pre-eclampsia are unclear and no marker predictive for the disease prior to clinical evidence of the disease has been identified. Furthermore an association has been observed between miscarriage and pre-eclampsia (Cooper *et al.*, 1988).

Epidemiological studies show the disease to be highly heritable, mainly confined to first pregnancies and largely prevented by normal first pregnancy by the same partner. Patients affected in first pregnancies have a 13.1% recurrence risk for their second, whereas with a normal first pregnancy, the incidence in the second is of the order of 1%. Thus, the first pregnancy appears to have a significant protective effect against pre-eclampsia in a subsequent pregnancy. Therefore, it follows that pre-eclampsia is preventable in principle (Lie *et al.*, 1998).

Several classification schemes have been proposed to aid clinical recognition of pre-eclampsia. The classification advocated by the US National Institutes of Health working group on hypertension in pregnancy, is a rise in blood pressure of >15mm Hg diastolic or >30mm Hg systolic from measurement in early pregnancy, or to >140/90 mm Hg in late pregnancy if no early reading is available; plus proteinuria (>0.3g per 24 h) and/or odema. However, in practice, proteinuria measurements may not always be determined and symptoms additional to a rise in blood pressure such as headache, visual disturbance and/or epigastric pain indicate a deterioration in pregnancy consistent with pre-eclampsia and form a basis for clinical intervention of early delivery by caesarean section to resolve the condition. Spinillo *et al.* (1994) reported that women with pre-eclampsia had a significantly increased incidence of intrauterine growth retardation (IUGR) small for gestational age (SGA) infants.

Although the cause of pre-eclampsia is unknown, hypertension is observed in pre-eclampsia and has been the focus of a large amount of research on the disorder. However, the pathological and physiological changes of pre-eclampsia show that this syndrome is much more than pregnancy-induced hypertension. Evidence to date implicates the action of placental trophoblasts as the underlying cause.

In pre-eclampsia, cytotrophoblast invasion is shallow and spiral arteriol invasion is abnormal, resulting in reduced blood perfusion of the intervillous space. Moreover the characteristic pattern of integrin switching that takes place during normal trophoblast differentiation does not occur in pre-eclampsia.

The outermost layer (trophoblasts) of the human placenta is devoid of classical class I human leukocyte antigens (HLA-A and HLA-B) and class II proteins (HLA-DR, HLA-DQ and HLA-DP). Although this prevents recognition by maternal T lymphocytes, the lack of class I molecules leaves these cells susceptible to attack by natural killer (NK) cells. However, trophoblast cells directly in contact with maternal tissues selectively express a characteristic nonclassical class Ib molecule, HLA-G, HLA-E and limited HLA-C expression also occurs . Expression of HLA-G has been shown to be sufficient to protect otherwise susceptible target cells from NK cell mediated lysis. NK cells usually express several different inhibitory receptors of various specificities at the same time. Cross linking of any single inhibitory receptor is sufficient to inactivate NK cell activity against all possible targets. It has been shown that membrane bound HLA-G molecules were able to inhibit alloreactive NK cells with NK inhibitory receptor 1 and inhibitory receptor 2 (NK1 and NK2). It has been shown that CD94 / NKG2 is the predominant inhibitory receptor involved in recognition of HLA-G by decidual and peripheral NK cells. Thus, at a functional level, HLA-G is able to protect target cells from destruction by NK1-,NK2-and NKG2 specific effector cells (Loke and King, 1997). More recently, HLA-G has been shown to modulate the ability of blood mononuclear cells to release cytokines (Maejima *et al.* 1997) suggesting a role for HLA-G in triggering maternal-foetal immune interplay. Specifically, coculturing of HLA-G expressing cells with

peripheral blood mononuclear cells (PBMC) increased the amount of interleukin-3 (IL-3) and interleukin-1 beta (IL-1 beta) and decreased the amount of tumour necrosis factor-alpha (TNF-alpha) release from the PBMC cells.

HLA-G binds a diverse but limited array of peptides in a manner similar to that found for classical class I molecules and it has been reported that HLA-G is expressed in the human thymus raising the possibility that maternal unresponsiveness to HLA-G expressing foetal tissues may be shaped in the thymus by central presentation of this MHC molecule on the medullary epithelium (Crisa *et al.* 1997) HLA-G is known to be capable of stimulating a HLA-G restricted cytotoxic T lymphocyte response and HLA-G molecules can serve as target molecules in lytic reaction with cytotoxic T lymphocytes and HLA-G expressed internally in vivo in transgenic animals is involved in education of the lymphocytic repertoire (Schmidt *et al.*, 1997).

Major histocompatibility (MHC) molecules bind a diverse array of peptides for presentation to T cells as part of a mechanism for recognition of self and non-self cells and pathologically altered cells. A detailed analysis of peptides bound to the soluble and membrane HLA-G proteins shows that, like MHC class I molecules, HLA-G also binds a diverse, although less complex array of peptides (Lee *et al.*, 1995). Some of these peptides, which are derived from intracellular proteins, constitute minor histocompatibility antigens which in conjunction with MHC molecules provoke an immune reaction by blood mononuclear cells such as T cells. HLA bound peptides can readily be fractionated, fully or partially purified and sequenced and can be assayed for their capacity to promote an immune reaction by measurement of their capacity to reconstitute lysis of target cells by cytotoxic T cells (den Haan *et al.*, 1998).

The entire gene sequence of HLA-G is known and DNA sequence analysis of HLA-G has shown that the HLA-G gene exhibits limited polymorphism. van der Van & Ober, 1995 examined the first six exons of HLA-G in 45 healthy African-Americans and observed variations in exons 2 and 3, which correspond to the alpha 1 and alpha 2 domains of the peptide binding grove. The most common polymorphism observed was a C to T transition at position 1488, corresponding to codon 93. Another common polymorphism was identified by Harrison *et al*, 1993 and is a 14 bp deletion in exon 8 of the gene. These results indicate that HLA-G is a polymorphic gene potentially capable of presenting a wide variety of peptides. Patterns of variability in HLA-G are similar to those of other class I MHC genes, where amino acid substitutions are clustered in the alpha 1 and alpha 2 domains.

Three observations of altered expression of HLA-G in pre-eclampsia have been reported. Colbern *et al.*, 1994 showed that the level of HLA-G in placental tissue was reduced in pre-eclampsia and that the decreased expression appeared to be related to a reduced number of trophoblasts in pre-eclamptic placental tissue. Hara *et al.*, 1996, showed that clusters of extravillous trophoblasts were devoid of HLA-

G in pre-eclamptic patients. Examination of human preimpantation blastocysts showed that only 40% of the blastocysts expressed HLA-G (Jurisicova, *et al.* 1996).

Inheritance

Several bodies of evidence show that pre-eclampsia and eclampsia are largely under genetic control. However the genetic mechanisms underlying susceptibility to pre-eclampsia remain unclear. This is largely due to confounding factors peculiar to its inheritance. First, the condition is specific to pregnancy and genetic studies to date have not been able to clarify whether the genes responsible are acting through the maternal or foetal genotype or through some interaction between the two. Secondly, pre-eclampsia is largely confined to primagravidas with a much lower incidence in subsequent pregnancies and thirdly, as the condition is specific to pregnancy, the genetic contribution of males is difficult to assess.

Diagnosis of true pre-eclampsia can be complicated by other hypertensive disorders such as essential hypertension and hypertension arising from renal disease. Such hypertensive disorders are distinct from true pre-eclampsia but nonetheless can confound diagnosis and thus pose problems for genetic studies.

The classification of pre-eclampsia by some investigators as a disease of immune dysfunction has prompted a number of studies on the role of the major histocompatibility complex in the genetics of pre-eclampsia.

There are numerous published studies on HLA associations with pre-eclampsia (Cooper *et al.*, 1993). Besides the fact that the positive associations are, with one exemption, not reproduced in studies by others, these studies suffer from other difficulties. The number of individuals are generally small in comparison to the large number of antigens at each of the HLA loci. There is a tendency for only significant associations to be reported and so there may be completed studies showing no association that have not been published besides those reported here. The first four associations reported are with antigen sharing or homozygosity. The number of antigens recognised has vastly increased with time. Antigens have been split as new sera become available, and the use of DNA techniques has split these further so that there are over 100 HLA-A and 100 HLA-B alleles and over 25 HLA-DRB alleles (including five different. common sequences recognised as DR4 serologically). Thus what were typed as the same allele in homozygotes or shared antigens in the early studies cannot be relied on to be homogenous in sequence or function. Detecting homozygotes with sera in early studies suffers from the extra difficulty of distinguishing them from heterozygotes for another allele for which sera did not exist (blanks).

At least three studies have further investigated the association between pre-eclampsia and HLA-DR by linkage analysis (Winton *et al.*, 1990; Hayward *et al.*, 1992, Harrison *et al.*, 1997). In these definitive studies no evidence was found for linkage of the HLA region to pre-eclampsia. Hayward *et al.* (1992)

also investigated several candidate genes and random DNA markers. Overall, no evidence was found for linkage to several candidate genes implicated in the pathogenesis of hypertension and their results excluded linkage to several markers. In these studies, an autosomal recessive model was assumed. Winton *et al.* (1990) also analysed their data for a HLA linkage using the affected sib pair method and the affected pedigree-member method. Both of these methods make no assumption about the mode of inheritance and neither gave any indication of linkage. The majority of pre-eclampsia cases are considered sporadic. A familial pregnancy-induced hypertensive disorder has been described and two loci have been implicated in the familial form of the disorder, namely, a candidate region on chromosome 4 and the eNOS gene region on chromosome 7 (Harrison *et al.*, 1997, Arngrimsson *et al.*, 1997). The epidemiology of PET is consistent with familial pregnancy-induced hypertensive disorder and sporadic PET being distinct entities.

Humphrey *et al.*, 1995, investigated the HLA-G deletion polymorphism for association with pre-eclampsia. Specifically, pre-eclamptic patients, offspring of pre-eclamptic mothers, blood relatives of pre-eclamptic patients, husbands of pre-eclamptic patients and a normal control group were genotyped for the polymorphism. The was no detectable association between pre-eclampsia in mothers or in offspring of pre-eclamptic mothers and the HLA-G deletion polymorphisms.

Karhukorpi et al., 1997 investigated HLA-G polymorphisms for association with recurrent spontaneous miscarriage. Specifically, they showed that there was no association between several HLA-G restriction fragment length polymorphisms and recurrent spontaneous miscarriage.

In the largest study of monozygotic twins, pre-eclampsia was reported in five first pregnancies, and all affected mothers were discordant with their twin. A second well documented report on an identical set of twins also showed clear discordance for pre-eclampsia in their first pregnancies. These observations argue against a recessive model and further support a role for the foetal paternal genotype in the disorder. Furthermore, although the subject of some controversy, pre-eclampsia occurs in mothers with mono-and di-zygotic twins arguing against a recessive foetal genotype and in favour of a dominant paternal gene in the foetus.

Some studies have considered the possibility of changing paternity as a contributing factor in the occurrence of pre-eclampsia in multiparae. Most notably, a strong association between pre-eclampsia and changing paternity has been observed (Lie *et al.*, 1998).

Much of the work on pre-eclampsia has been based on the hypothesis of a major susceptibility locus in the affected mother and almost all of the genetic studies to date have focused on linkage or association between the genotype of the mother and pre-eclampsia. In order to test the hypothesis that foetal HLA-G

is the most likely candidate gene for the disorder, we have investigated HLA-G genotypes in pre-eclamptic and control trios and have shown that HLA-G is linked to both normal and pre-eclampsia pregnancy outcome and associated with recurrent spontaneous abortion. We have also investigated HLA-G genotypes in second pregnancies of control and pre-eclamptic trios and have shown that the presence of specific HLA-G alleles in the foetus in first pregnancy permits the occurrence of different HLA-G alleles in second pregnancy showing that HLA-G can induce tolerance to antigens in the first pregnancy and/or can modify the maternal immune system to accept foetuses in the second pregnancy in the absence of pregnancy related disorders that are selected against and/or cause pregnancy related disorders in first pregnancy.

Early pregnancy loss is the most common complication of human gestation of women attempting pregnancy. The majority of these losses are clinically unrecognised. Using a highly sensitive assay, the total incidence of miscarriage was estimated to be 31%, including 22% of losses which occurred at the very early stages of pregnancy i.e. before the pregnancy was clinically recognised (Wilcox *et al.*, 1988). Recurrent spontaneous abortion (RSA) or recurrent miscarriage, defined as the loss of three or more spontaneous pregnancies before 20 weeks gestation, occurs in less than 1% of pregnant women. Studies suggest that the chance of a successful pregnancy in an untreated woman who has experienced two or more first trimester miscarriages and no live births is approximately 30% to 50%. It is generally accepted that RSA is a condition with many different causes, however, about 50% of all RSA cases are not explained by structural genetic, endocrine, infectious or anatomic factors. Within the past few years there has been a growing recognition that recurrent pregnancy loss may have autoimmune (immunity against self) and alloimmune (immunity against another person) causes, even in women with no clinically diagnosed autoimmune diseases. This has lead to investigation of the role of the HLA system and RSA, in particular, much emphasis has been placed on the degree of sharing of HLA alleles and haplotypes between RSA couples. It has been suggested that fetuses whose HLA alleles do not differ from maternal alleles (i.e. histocompatible fetuses) are more likely to be aborted than fetuses with HLA alleles that differ from maternal alleles (i.e. histoincompatible fetuses). It would follow then that couples who match for HLA alleles or haplotypes would produce histocompatible fetuses and hence be at risk of miscarriage.

Ober *et al.* (1998) conducted a 10 year prospective study of HLA matching and pregnancy outcome. A significant increase in fetal loss was observed in couples who matched for a 16-locus haplotype encompassing the entire HLA locus. Christiansen *et al.* (1997) examined HLA-C and HLA-Bw in unexplained RSA couples. They found no variation in HLA-C, but a significantly higher number of RSA couples have the HLA-Bw4 haplotype than control couples. Jin *et al.*, (1995) examined the degree of sharing of HLA-A, HLA-B, HLA-DR and HLA-DQ haplotypes. They found a significant excess of

HLA-DR sharing in couples with RSA, and also a significant excess of HLA-DQ sharing in couples with unexplained infertility.

Several groups have recorded conflicting results. Billingard *et al.*, (1995) examined sharing of HLA-A, HLA-B, and HLA-DR alleles and found no higher degree of HLA sharing in couples with RSA than in fertile couples. Caudle *et al.*, (1983) reported similar findings. HLA-A, HLA-B and HLA-DR alleles were typed in a large population of unexplained RSA couples and in control couples (Sbracia *et al.*, 1996). No increased sharing in HLA alleles was observed. In addition, there was no difference in the frequency of HLA alleles between RSA couples and control couples. Saski *et al.*, (1997) reported an increase in the frequency of the HLA-DR4 allele in women who suffered from RSA compared to control women.

The role of HLA sharing as a risk factor for RSA remains controversial, and no studies have reported any diagnostic or prognostic significance to HLA sharing in individual couples. In addition, reports of significant sharing of class II genes are difficult to explain as fetal cells in contact with the maternal immune system during pregnancy are devoid of HLA class II expression.

According to the present invention there is provided a method for diagnosing susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility comprising the steps of:

a) obtaining a fluid and/or tissue sample from a female and/or male and/or foetus; and either

b) determining the sequence of all or part of the HLA-G nucleic acid, and/or HLA-G linked nucleic acid; or

c) detecting variant forms of all or part of the HLA-G protein, and/or proteins encoded by HLA-G linked genes or:

d) measuring the functional activity of all or part of the HLA-G encoding protein and/or proteins encoded by HLA-G linked genes or:

e) measuring the size and/or level of all or part of HLA-G mRNA or mRNA transcribed from HLA-G linked genes or:

f) measuring the size and/or level of all or part of HLA-G protein and/or protein encoded by HLA-G linked genes or:

g) quantifying cells or molecules whose concentration changes as a result of HLA-G action; and

h) comparing any of the parameters b) to g) with those of a female and/or male and/or foetus of a normal pregnancy and/or a pregnancy with pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related fertility outcome.

Preferably the HLA-G nucleic acid is analysed by the presence of the C and/or T allele of codon 93 in exon 3 and/or the insertion and/or deletion allele of exon 8.

Preferably the effect of one or more of the HLA-G sequence variants on the functional activity of HLA-G and/or on the size and/or the level of all or part of the HLA-G mRMA and/or its encoded peptide is measured.

In its simplest the present invention provides a method of diagnosing susceptibility to pre-eclampsia and/or eclampsia and/or intrauterine growth retardation a nd/or susceptibility to miscarriage and/or miscarriage-related infertility comprising the steps of:

a) obtaining nucleic acid from a parent and/or a prospective parent and/or foetus;

b) establishing the HLA-G sequence variants present in the parent and/or foetus by analysing the nucleic acid isolated in step (a); and

c) comparing the HLA-G sequence variants identified in step (b) with known HLA-G sequence variants.

Preferably, the HLA-G sequence variants are established by characterising all or part of the DNA sequence of the HLA-G gene by methods selected from DNA sequencing, PCR-restriction fragment length polymorphism analysis, glycosylase mediated polymorphism detection, oligonucleotide hybridisation, gel electrophoretic detection of polymorphisms and amplification based detection approaches.

Suitably, a stratified approach is used whereby the C/T-93 in exon 3 and insertion/deletion polymorphism in exon 8 are first genotyped, followed by genotyping of other variations in exon 3, exon 2, intron 2, followed by exon 1 and 4, followed by the remainder of the HLA-G gene.

The invention also provides a test kit for the diagnosis of susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility comprising:

a) oligonucleotide primers for amplification of all or part of the HLA-G gene and/or HLA-G linked DNA;

b) amplification reagents for amplification of genomic DNA and/or RNA segments, selected from a DNA / RNA polymerase, a reverse transcriptase, the deoxyribonucleotides dATP, dCTP, dGTP, dTTP and dUTP. and/or ribonucleotides ATP, CTP, GTP, TTP and UTP, and reaction buffer;

c) reagents for identifying sequence variants in DNA and/or RNA;

d) control DNA and/or RNA.

Preferably the primers of (a) allow specific amplification of all or part of the HLA-G gene using the polymerase chain reaction. Several polymorphisms are known to occur in the HLA-G gene. A C to T

polymorphism occurs at nucletide 1488 in the third position of codon 93 and is referred to as C/T-93 herein where C-93 is one allele of the polymorphism and T-93 is the other allele of the polymorphism. Suitably, the C/T-93 polymorphism is genotyped by PCR amplification of a section of intron 2 - exon 3 using the primers 5'-TACTCCCGAGTCTCCGGGTCTG-3'(SEQ ID NO. 1) as the forward primer and 5'-AGGCGCCCCACTGCCCCTGGTAC-3'(SEQ ID NO. 2) as the reverse primer giving rise to the amplified C-93 allele (SEQ ID NO. 4) and amplified T-93 allele (SEQ ID NO. 5) followed by by semi-nested PCR amplification using the forward primer 5'-GACCGAGGGGGTGGGGCCAGGTTCT-3'(SEQ ID NO. 3) and the reverse primer 5'-AGGCGCCCCACTGCCCCTGGTAC-3'(SEQ ID NO. 1). In the semi-nested amplification reaction dTTP is replaced by dUTP. The 3' end of the forward primer is designed so that the first U incorporated downstream of the forward primer is at, or distal to, the polymorphic site in codon 93. Following amplification using end labelled forward primer, glycosylase mediated cleavage of the amplified product is performed. Cleavage products are resolved by denaturing gel electrophoresis (20% polyacrylamide) and visualised by autoradiography. The C-93 allele is detected as a 32 n fragment (SEQ ID NO. 8)and the T-93 allele as a 27 n fragment (SEQ ID NO. 9).

The common 14 base pair insertion / deletion polymorphism in exon 8 of the HLA-G gene is referred to as I/D-E8 herein where I-E8 is one allele of the polymorphism and D-E8 is the other allele of the polymorphism. Suitably, genotyping of the HLA-G exon 8 deletion polymorphism is performed by amplifying a short section flanking the deletion location in exon 8. This is achieved using the polymerase chain reaction with primers designed to hybridise to known DNA sequence in exon 8. The forward primer is 5'-TGTGAAACAGCTGCCCTGTGT-3' (SEQ ID NO. 10) and the reverse primer is 5'-AAGGAATGCAGTTCAGCATGA-3' (SEQ ID NO. 11). The I/D exon 8 polymorphism is genotyped by size separation of the PCR products on a 10% non denaturing polyacrylamide gel and visualised by staining with ethidium bromide, the I-E8 insertion allele giving rise to a 151 bp product (SEQ ID NO. 12) and the D-E8 deletion allele giving rise to a 137 bp product (SEQ ID NO. 13).

Suitably, allele specific genotyping is performed in cases where maternal and paternal C/T-93 and I/D-E8 HLA-G haplotypes cannot be directly assigned. This is achieved using allele specific primers which allows selective amplification of the I-E8 or D-E8 allele. Following allele specific amplification, the C/T-93 polymorphism is then genotyped using the GMPD assay described above. Primers for amplification of the I-E8 allele are 5'-TACTCCCGAGTCTCCGGGTCTG-3' (SEQ ID NO. 1) as the forward primer and 5'-CAAAGGGAAGGCATGAACAAATCTTG-3' (SEQ ID NO. 14) as the reverse primer. Primers for amplification of the D-E8 allele are 5'-TACTCCCGAGTCTCCGGGTCTG-3' (SEQ ID NO. 1) as the forward primer and 5'-GTTCTTGAAGTCACAAAGGGACTTG -3' (SEQ ID NO. 15) as the reverse primer. Such allele specific amplification gives rise to four possible haplotypes, namely I-E8 and C-93

haplotype (SEQ ID NO. 16), I-E8 and T-93 haplotype (SEQ ID NO. 17), D-E8 and C-93 haplotype (SEQ ID NO. 18) and D-E8 and T-93 haplotype (SEQ ID NO. 19).

Suitably, haplotypes are constructed and suitably, transmitted and non-transmitted alleles to offspring / foetus are assigned.

Preferably, the amplification reagents include a thermostable DNA polymerase, amplification buffer and DNA precursor nucleotides.

All or part of any HLA-G sequence and/or HLA-G linked sequence may also be amplified, by a method or combination of methods selected from nucleic acid sequence based amplification, self-sustained sequence replication, transcription-mediated amplification, strand displacement amplification and the ligase chain reaction.

Preferably the comparison of one or more variants identified is performed by association and/or linkage analaysis and/or transmission analysis. Preferably all or part of the HLA-G sequence is cloned into a vector.

The invention may involve:

a) obtaining nucleic acid or fluid or tissue sample from a parent and/or prospective parent and/or foetus;

b) establishing the HLA genotype or serotype of the parent/prospective parent and/or foetus by analysing the nucleic acid or fluid or tissue sample isolated in step (a);

c) comparing the HLA genotypes or serotypes identified in step (b) with known HLA genotypes or serotypes respectively.

Preferably the method involves the measuring of cellular and/or soluble HLA-G levels. Preferably HLA-G levels are measured by immunoassay using an antibody for specific HLA-G protein.

The invention may involve identifying the variant form of HLA-G protein and/or the levels thereof present in the sample. Preferably HLA-G variant proteins and/or levels thereof are detected and/or quantified by immunoassay using specific antibodies which detect HLA-G variants, or HLA-G protein. Alternatively, antibody specific for HLA-G protein variants and/or electrophoretic separation methods and/or chromatographic separation methods may be used. Preferred methods for detecting HLA-G protein and variants thereof include, enzyme linked immunosorbent assays (ELISA), radioimmuno-assays (RIA), immunoradiometric assays (IRMA) and immunoenzymatic assays (IEMA), including sandwich assays using monoclonal and/or polyclonal antibodies.

The invention may involve measuring of level of molecules whose concentration changes as a direct and/or indirect result of HLA-G action. Preferably the molecules are selected from IL-1, IL-2, IL-3, IL-4, IL-6, IL-10 beta and tumour necrosis factor alpha. Preferably the levels of such molecules are measured by immunoassay using antibodies specific for the molecules.

Alternatively, the method may involve measuring of levels of trophoblast specific markers. Preferably the trophoblast markers are cytokeratins pregnancy specific glycoprotein 1, human chorionic gonadotrophin and human placental lactogen. Preferably the levels of such molecules are measured by immunoassay using antibodies specific for the molecules.

In one embodiment the method may comprise the steps of:

a) incubating blood mononuclear cells and/or a subset of such cells with one or more HLA-G variants and/or any combination thereof and/or cells expressing all or part of one or more variants of the HLA-G gene and/or a combination of one or more variants thereof, wherein the blood mononuclear cells and/or HLA-G variant is from a female and/or male and/or foetus

b) analysing the activity of the blood mononuclear cells and/or the HLA-G and/or cells expressing one or more HLA-G variant.

Preferably, the blood mononuclear cells are obtained as a blood sample and/or tissue sample from the female and/or are obtained through matching the females blood mononuclear cells with blood mononuclear cells from a donor and/or cell line panel. Preferably populations of T cells and/or NK cells are isolated from the blood sample by density centrifugation and/or immunoselection. Preferably, blood mononuclear cells matching the females blood mononuclear cells are identified from a test panel by matching the HLA serotype and/or extended HLA genotype and/or HLA-G genotype of the female with the HLA serotype and/or extended HLA genotype and/or HLA-G genotype of blood mononuclear cells. Preferably, HLA-G matching the male and/or female HLA-G is identified from a test panel by matching the HLA-G type and/or HLA-G genotype of the male and/or female with the HLA-G type and/or HLA-G genotype of HLA-G proteins and/or cells expressing one or more HLA-G gene variants in the test panel. Preferably, such a test panel is assembled by growing cells expressing one or more HLA-G variants. Such cells may be derived from natural tissue such as placenta and/or created artificially by the introduction of one or more vectors bearing HLA-G gene variants which are capable of promoting the expression of the HLA-G gene into a cell and/or by inducing the expression of native HLA-G in cells. Suitably, the vector used is plasmid, phage, viral, and/or artificial chromosome based. Preferably HLA-G protein is used as a crude preparation and/or fully or partially purified from such cells. HLA-G protein may be loaded with binding peptides naturally or artificially.

Preferably, the HLA-G - blood mononuclear cell interaction is measured by assessing blood mononuclear cell activation including assessment of one or more of the following; cell proliferation, transformation cytotoxic response, surface marker expression, cytokine production, conjugate formation and target specificity.

The method may comprise the steps of:

a) cloning the HLA-G gene from a parent and/or prospective parent and/or foetus;

b) expressing the HLA-G protein from the cloned gene *in vitro* and/or *in vivo*;

c) measuring the levels of activity of the expressed HLA-G protein;

d) comparing the levels of activity of the expressed HLA-G protein with the levels of activity observed for the normal HLA-G protein.

The method may also comprise:

a) establishing all or part of the HLA-G sequence and/or HLA-G linked sequences present in a sample from a female and/or male and/or foetus by analysing the nucleic acid from said sample;

b) determining whether one or more of any variants or any combination thereof, identified in step (a) are indicative of susceptibility to normal pregnancy or pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility by comparative analysis and/or analysis of the effect of one or more of the variants on the functional activity of HLA-G and/or on HLA-G mRNA.

Preferably, the HLA-G sequence variants are established by characterising all or part of the DNA sequence of the HLA-G gene and/or closely linked DNA including HLA-A, HLA-B, HLA-C, HLA-E, HLA-F and HLA-H genes by amplifying all or parts HLA-G or closely linked DNAs and identifying the sequence variants present using one or more sequence variation detection methods.

Suitably, one or more copies of all or parts of the HLA-G gene is amplified by any of several amplification approaches such as the polymerase chain reaction (PCR), nucleic acid sequence based amplification (NASBA), self sustained sequence replication (3SR), transcription-mediated amplification (TMA) and strand displacement amplification. Amplification of a target nucleic acid molecule may also be carried out using a the ligase chain reaction (LCR) and a variation of the LCR which employs a short PCR step (PLCR). Suitably, DNA or mRNA is used as the amplification substrate. Suitably, mRNA is converted into DNA using reverse transcriptase. Suitably, the amplified molecules are analysed directly and/or may be cloned into a vector to facilitate analysis. Suitably, DNA sequence variations are detected by any one or more of a variety of gene variation detection methods including DNA sequencing, glycosylase mediated polymorphism detection, restriction fragment length polymorphism analysis, enzymatic or chemical cleavage assays, hybridisation to DNA probe arrays, allele specific oligonucleotide hybridisation assays, allele specific amplification methods such as the amplification refractory method

(ARMS), electrophoretic detection of polymorphisms based on migration through a gel matrix, 5' nuclease assay and ligase chain reaction.

Suitably, it can be determined if one or more variants identified are known variants associated with susceptibility to normal pregnancy and/ or susceptibility to pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility. Alternatively, comparative analysis is performed by gene association and/or gene linkage methods to determine whether HLA-G variants and/or HLA-G linked variants are associated with normal pregnancy and/ or susceptibility to pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility.

Alternatively, HLA-G variants associated with normal pregnancy and/ or susceptibility to pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility can be identified by the effect of HLA-G variants on HLA-G function. Suitably, HLA-G variants are functionally analysed by measuring the interaction of one or more of the HLA-G variants and/or any combination thereof, with blood mononuclear cells and/or measuring the size and level of the HLA-G messenger RNA and/or the size and level of HLA-G gene product and/or peptide binding for one or more of the HLA-G variants and/or any combinations thereof. HLA-G - blood mononuclear cell activity is measured by assessing blood mononuclear cell activation including assessment of one or more of the following; cell proliferation, cytotoxic response, surface marker expression, cytokine production, conjugate formation and target specificity.

The invention also relates to a pharmaceutical composition comprising a pharmaceutically effective amount of HLA-G protein and/or cells expressing HLA-G and/or one or more peptides which binds to HLA-G and/or blood mononuclear cells from a donor and/or a cells from a test panel known to interact with HLA-G variants, cytokines and any combination thereof including IL-1 beta , IL-2, IL-3, IL-4, IL-6, IL-10 and tumour necrosis factor-alpha and/or inhibitors of cytokines and/or tumour necrosis factor alpha and/or derivatives of cytokines and/or tumour necrosis factor-alpha, optionally with pharmaceutically-acceptable carriers or excipients.

The invention also provides a method for screening for agents which can potentially be used as diagnostic indicators and/or drug targets for pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation by:

a) measuring the expression level of one or more genes and / or proteins in HLA-G expressing cells and /or blood mononuclear cells and / or T cell and /or natural killer cell subsets thereof following interaction with HLA-G and / or HLA-G expressing cells;

b) comparing the expression level identified in step (a) with the expression level in HLA-G expressing cells and /or the blood mononuclear cells and / or T cell and /or natural killer cell subsets thereof following interaction with HLA-G and / or HLA-G expressing cells in normal pregnancy and/ or pre-eclampsia pregnancy and/or intrauterine growth retardation pregnancy and/or miscarriage pregnancy and/or miscarriage-related infertility.

In a further aspect the invention provides a method for screening for potential pre-eclampsia and eclampsia and intrauterine growth retardation and miscarriage and miscarriage-related infertility therapeutic agents selected from:

a) identifying agents which alter the expression of HLA-G;

b) identifying agents which alter the activity of HLA-G;

c) identifying agents which mimic the action of HLA-G;

d) identifying agents which bind to HLA-G;

e) identifying peptides which bind to HLA-G;

f) identifying agents which bind to HLA-G receptors;

g) identifying expressed genes using DNA probe arrays in a cellular background in HLA-G expressing cells and/or blood mononuclear cells interacting with HLA-G and/or cells expressing HLA-G interacting with blood mononuclear cells;

h) identifying expressed genes using DNA probe arrays in a cellular background whose expression is altered in response to HLA-G expression in the cells and/or in response to interacting cells expressing HLA-G;

i) identifying expressed proteins using mass spectrometry methods in HLA-G expressing cells and/or blood mononuclear cells interacting with HLA-G and/or cells expressing HLA-G interacting with blood mononuclear cells.

Preferably sperm and/or semen and/or female reproductive tissue are screened for agents:

a) which alter the expression of HLA-G in fertilised eggs and/or embryos;

b) which alter the cell cleavage rate of fertilised eggs and/or embryos;

c) which induce cellular factors in cell in culture and/or cell in vivo that alter the cell cleavage rate of fertilised eggs and/or embryos.

The method may involve:

a) measuring the expression level of one or more genes and/or proteins in HLA-G expressing cells and/or blood mononuclear cells and/or T cell and/or natural killer cell subsets thereof following interaction with HLA-G and/or HLA-G expressing cells;

b) comparing the expression level identified in step (a) with the expression level in HLA-G expressing cells and/or the blood mononuclear cells and/or T cell and/or natural killer cell subsets thereof following interaction with HLA-G and/or HLA-G expressing cells associated with normal pregnancy and/ or pre-

eclampsia pregnancy and/or intrauterine growth retardation pregnancy and/or miscarriage pregnancy and/or miscarriage-related infertility.

Preferably, blood mononuclear cells and/or HLA-G expressing cells are obtained from a female and/or male and/or foetus and/or test panel of blood mononuclear cells and/or HLA-G expressing cells.

Preferably, gene expression is measured by any one or combination of several methods including hybridisation between cDNA and/or RNA from the cells and DNA probes and/or RNA probes and/or DNA probe arrays, quantitative amplification approaches such as quantitative (reverse transcriptase - polymerase chain reaction) RT-PCR, 5' nuclease assay, ribonuclease protection assay and S1 nuclease assay.

Preferably, protein expression is measured by any one or combination of several methods including one dimensional and/or two dimensional gel electrophoresis and staining of proteins and/or detection of one or more proteins using, enzyme linked immunosorbent assays (ELISA), radioimmunoassays (RIA), immunoradiometric assays (IRMA) and immunoenzymatic assays (IEMA), including sandwich assays and Western blotting using monoclonal and/or polyclonal antibodies.

Alternatively, the method may involve:

a) measuring the expression level of one or more genes and/or proteins in cells expressing HLA-G; and

b) comparing the expression level identified in step (a) with the expression level in HLA-G non-expressing cells.

Preferably, the cells are fertilised animal eggs and/or animal embryos.

The invention also provides a method for the prevention of pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility selected from:

a) treatment of a female with all or part of a pharmaceutically effective amount of an effective HLA-G protein and/or peptides which bind to HLA-G and/or cells expressing HLA-G;

b) treatment of a female with all or part of a pharmaceutically effective amount of molecules or inhibitors of molecules whose level or activity is directly or indirectly altered by HLA-G;

c) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters HLA-G expression;

d) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters NK cell activity;

e) treatment of a female with all or part of a pharmaceutically effective amount of an agent which mimics all or part of HLA-G action:

f) treatment of a female with all or part of a pharmaceutically effective amount of molecules which inhibit the interaction between HLA-G and one or more of its receptors;

g) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters the size and/or level of HLA-G mRNA;

h) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters HLA-G related blood mononuclear cell activity;

i) treatment of a female with blood mononuclear cells that recognise foetal and/or self HLA-G;

j) treatment of a female with HLA-G protein and/or cells expressing HLA-G.

The invention may comprise:

a) obtaining blood mononuclear cells and/or T cell and/or natural killer cell subsets thereof and/or HLA-G and/or HLA-G expressing cells from a female and/or male and/or foetus and/or test panel;

b) measuring the expression level of one or more genes and/or proteins in the HLA-G expressing cells and/or blood mononuclear cells following interaction with HLA-G and/or HLA-G expressing cells;

c) comparing the expression level identified in step (b) with the expression level in the blood mononuclear cells and/or HLA-G expressing cells in normal pregnancy and/ or pre-eclampsia pregnancy and/or intrauterine growth retardation pregnancy and/or miscarriage pregnancy and/or miscarriage-related infertility.

Preferably, the blood mononuclear cells and/or HLA-G expressing cells are obtained as a blood sample and/or tissue sample. Preferably populations of T cells and/or NK cells are isolated from the blood sample by density centrifugation and/or immunoselection. Preferably HLA-G expressing cells are isolated by immunoselection.

The invention also provides a method for improving fertility and pregnancy outcome wherein male and/or female partners and/or sperm and/or ova and/or recipients of fertilised eggs and/or zygotes / and/or embryos are selected on the basis of HLA-G so that their genotypes and/or serotypes are associated with normal pregnancy outcomes and/or not associated with pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility.

In particular there is provided a method for improving pregnancy success selected from:

a) pre-treating the female with sperm and/or attenuated forms thereof, and/or semen and/or fractions thereof from a male with a known HLA-G genotype, prior to mating with a male of a different HLA-G genotype, and/or in vitro fertilisation using sperm from a male of a different HLA-G genotype and/or embryo transfer where the male HLA-G is of a different HLA-G genotype;

b) mixing sperm of a known HLA-G genotype with sperm and/or attenuated forms thereof, and/or semen and/or fractions thereof from a male with a different HLA-G genotype prior to in vitro fertilisation.

Preferably fertility and/or pregnancy outcome are improved by selection of male and / or female partners and / or sperm and / or ova and / or recipients of fertilised eggs and / or zygotes / and / or embryos so that (a) their HLA-G and /or HLA genotypes and /or serotypes or (b) the activity of their HLA-G and / or blood mononuclear cells interacting with HLA-G are indicative of normal pregnancy outcomes and / or not associated with pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility.

Cloning of all or part of one or more HLA-G genes in any of the above methods may be achieved by amplification of all or part of one or more HLA-G genes and insertion of all or part of the amplified product into a vector capable of expressing the inserted gene. Expression of the HLA-G protein from the cloned gene in any of the above methods may be achieved by introduction of the expression vector into a suitable host such as a bacterium or an eukaryotic cell in culture. The level of activity of the expressed HLA-G protein in any of the above methods may be achieved by a) directly and/or indirectly measuring the interaction of the HLA-G protein and/or cells expressing HLA-G protein with blood mononuclear cells and/ or b) detecting one or more molecules whose level is altered as a result of the interaction of the HLA-G protein and/or cells expressing HLA-G protein with blood mononuclear cells and/or c) measuring changes in cell cleavage rate due to direct and/or indirect action of the HLA-G protein and/or cells expressing HLA-G protein with blood mononuclear cells.

HLA-G as defined herein refers to any form of HLA-G and / any complex involving HLA-G including different isoforms of HLA-G arising from alternative splicing pathways, combination of different HLA-G isoforms, secreted HLA-G, membrane bound HLA-G HLA-G with peptides bound and HLA-G associated with beta -2-microglobulin. HLA-G protein refers to any crude, partially and/or fully purified form of HLA-G.

The invention also provides use of a DNA sequence selected from any one of sequence I.D.s 1 to 21 for diagnosis of susceptibility to or in a test kit for the diagnosis of susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility, for monitoring progress of pregnancy, for use in the manufacture of a medicament. in a method for screening potential therapeutic agents, in a method for screening for potential diagnostic indicators and/or drug targets, in a method for improving pregnancy success or in a method for the prevention of pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility, for monitoring progress of pregnancy.

The invention also provides a method for induction of tolerance in a host to a non-self tissue which comprises administering HLA-G and /or HLA-G loaded with peptides from the non-self tissue and /or

HLA-G expressing cells derived from or related to the non-self tissue, and/or a non-self tissue bearing an introduced HLA-G gene so that HLA-G is expressed in all or part of the tissue.

In a further aspect the invention provides a method for the treatment of autoimmune disease which comprises administering HLA-G and /or HLA-G loaded with peptides from a self or non-self tissue and / or with specific autoimmune antigen and /or HLA-G expressing cells from a self and/or non-self tissue and/or a self and/or non-self tissue bearing an introduced HLA-G gene so that HLA-G is expressed in all or part of the tissue.

Methods

Identification of Subjects

In the initial phase of sampling pre-eclamptic patients were identified as primagravidas who were delivered by caesarean section at or prior to 36 weeks gestation because of a deterioration in pregnancy indicative of pre-eclampsia. Diagnostic symptoms were a rise in blood pressure >15mm Hg diastolic or >30mm Hg systolic from measurement in early pregnancy or to >140/90mm Hg in late pregnancy, and one or more of the following: proteinuria, odema, headache, visual disturbance, epigastric pain. Control patients were identified as primagravidas with normal delivery and normal blood pressure. 5-10mls of blood were taken from the offspring of primagravida pre-eclampsia and normal pregnancies with informed consent.

In the second phase of sampling, blood samples and/or cheek swab sample for DNA extraction were collected from control trios following delivery. The appropriate informed consent was obtained from all subjects. Control mothers were identified as primagravidas under the age of thirty three with normal delivery and normal blood pressure. All individuals were Irish and Caucasian by origin. Mothers were interviewed to ensure that they were primagravidas. Primagravida (first pregnancy) pre-eclampsia trios where the mothers suffered severe pre-eclampsia and a matching control group of normal primagravida trios were identified and sampled. Families (mother, father, first and second offspring) where the mother had two or more successful normal pregnancies in the absence of pregnancy related disorders including pre-eclampsia and miscarriage were also identified and sampled. Families (mother, father, first and second offspring) where the mother had pre-eclampsia in the first pregnancy and a normal second pregnancy in the absence of pregnancy related disorders including pre-eclampsia and miscarriage were also identified and sampled. Couples with recurrent spontaneous abortion were also identified and sampled. To minimise the possibility of misdiagnosis of PE, we applied stringent criteria to ascertainment of samples. Essentially pre-eclampsia cases were identified as primagravidas under the age of 35 who were delivered by caesarean section at, or prior to, 36 weeks gestation because of a deterioration in pregnancy indicative

of pre-eclampsia. Diagnostic symptoms were a rise in blood pressure of >15mm Hg diastolic or >30mm Hg systolic from measurement in early pregnancy or to >140/90mm Hg in late pregnancy, and one or more of the following: proteinuria, odema, headache, visual disturbance, epigastric pain. Diagnostic symptoms were completely resolved within 3 months after delivery. A preliminary survey of the sisters of the pre-eclamptic women in this study did not reveal an increased incidence of the condition, indicating that pre-eclampsia in the cohort of mothers investigated here is sporadic. A cohort of couples where the mother had three or more consecutive miscarriages were identified (recurrent miscarriage).

Genotyping of HLA-G polymorphism

Genomic DNA was extracted from peripheral blood samples and/or cheek swab samples by standard methods. DNA concentration was determined by absorbance at 260nm for samples where DNA was isolated from blood. The integrity and purity of the genomic DNA was determined by agarose gel electrophoresis and OD260:OD280 ratio respectively.

The C-93T HLA-G polymorphism is also known as the C/T codon 93 polymorphism (and as HLA-G C1488T) and referred to as C/T-93 herein where C-93 is one allele of the polymorphism and T-93 is the other allele of the polymorphism. In order to genotype the C/T-93 polymorphism in the genomic DNA samples, exon 3 of the HLA-G gene was first amplified using the polymerase chain reaction with primers designed to hybridise to the known DNA sequence flanking exon 3. The forward primer was 5'-TACTCCCGAGTCTCCGGGTCTG-3' (SEQ ID NO. 1) and the reverse primer was 5'-GAGGCGCCCCACTGCCCCTGGT-3'.

The polymerase chain reaction was carried out in a total volume of 25µl, with 100ng genomic DNA, 50ng of each primer, 0.2mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP and dTTP) 50mM KCl, 10mM Tris-HCl, pH 9.0 at 25°C, 0.1% Triton X-100, 1.5mM MgCl and 0.5U of Taq Polymerase. Reaction mixtures were covered with an equal volume of mineral oil and amplification was carried out using the "hot start" technique in a thermal cycler. The conditions for amplification involved denaturation at 94 ^{0}C for 5 min followed by addition of Taq Polymerase. Thirty cycles were then performed: 94 °C for 1 min, 63°C for 1 min, 72°C for 1 min and finally a 10 min extension at 72°C.

Genotyping of the C/T-93 HLA-G polymorphism was then performed using a semi nested amplification approach and the Glycosylase Mediated Polymorphism Detection method (Vaughan and McCarthy 1998). A 319bp section of the HLA-G gene encompassing the C/T-93 polymorphism location was amplified using a semi nested polymerase chain reaction approach from the previously amplified exon 3 of the HLA-G gene using the exon 3 reverse primer and the internal forward primer 5'-GACCGAGGGGGTGGGGCCAGGTTCT-3' (SEQ ID NO. 3). The forward primer was end labelled by

incubation with polynucleotide kinase in the manufacturers buffer (New England Biolabs) and 5μCi 32P-ATP (3000Ci/mmol) for 30 min at 37°C followed by ethanol precipitation to remove unused labelled nucleotide. The semi nested amplification reaction was carried out in a total volume of 10μl, with 1μl of a 1 in 500 dilution of the previously amplified exon 3 product, 3pmoles of forward and reverse primer, 0.2mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP and dUTP) 50mM KCl, 10mM Tris-HCl, pH 9.0 at 25°C, 0.1% Triton X-100,1.5mM MgCl and 0.5U of Taq Polymerase. Reaction mixtures were covered with an equal volume of mineral oil and amplification was carried out using the "hot start" technique in a thermal cycler. The conditions for amplification involved denaturation at 94°C for 5 min followed by addition of Taq Polymerase. Thirty cycles were then performed: 94°C for 1 min, 64°C for 1 min, 72°C for 1 min and finally a 10 min extension at 72°C. The reaction mixture was then treated with exonuclease I to digest the primers not extended in the amplification step. This was achieved by incubating the PCR reaction mixture with 0.4 units of exonuclease I at 37°C for 30 min. The exonuclease was subsequently heat inactivated by incubating the reaction at 80°C for 15 min.

Uracil DNA-glycosylase (0.5 units) was then added and the incubation continued at 37°C for min. Following treatment with uracil DNA-glycosylase, the AP sites generated in the amplified product were cleaved to completion by adding NaOH to a final concentration of 0.05M and heating the mixture for 15 min at 95°C. Under these conditions, cleavage occurs on the 5' side of each AP site. The reaction was then neutralised by addition to Tris base to 30mM final concentration. Both Exol and UDG are diluted containing 0.07M Hepes KOH pH 8.0, 1mM EDTA, 1mM DTT and 50% glycerol.

An equal volume of formamide loading dye (90% formamide, 0.025% Bromophenol blue, 0.025% Xylene cylanol) was added to the sample which was then heated at 85°C for 5 min. The sample was then loaded onto a 20% denaturing (7M urea) polyacrylamide gel and electrophoresis was carried out for 3-4 hours at 60W for size analysis of the cleaved products in the sample. Following electrophoresis, autoradiography was carried out by exposing the gel directly to X-ray photographic film for 12 hrs at -70°C. During the second phase of the genotyping, an improved protocol was used. Essentially, PCR amplification was carried out in 25 ml reactions, each of which contained 100 ng genomic DNA, PCR buffer (100 mMTris-HCl pH 8.3 (20°C), 500 mM KCl, 15 mM $MgCl_2$), 200 mM of each dNTP, 300 nM of each primer and 0.5 U *Taq* polymerase (Boehringer). Conditions for amplification of exon 3 were 30 cycles at 94°C for 45 s, 61°C for 45 s, 72°C for 60 s using 5'-TACTCCCGAGTCTCCGGGTCTG-3'(SEQ ID NO. 1) as the forward primer and 5'-AGGCGCCCCACTGCCCCTGGTAC-3'(SEQ ID NO. 2) as the reverse primer giving rise to the amplified C-93 allele (SEQ ID NO. 4) and amplified T-93 allele (SEQ ID NO. 5). All of the samples were then genotyped for the HLA-G C/T-93 polymorphism using the recently described glycosylase mediated polymorphism detection (GMPD) method (Vaughan & McCarthy, 1998).

Essentially a 319 bp fragment was amplified by semi-nested PCR from exon 3 using the forward primer 5'-GACCGAGGGGGTGGGGCCAGGTTCT-3'(SEQ ID NO. 3) and the reverse primer 5'-AGGCGCCCCACTGCCCCTGGTAC-3'(SEQ ID NO. 1) giving rise to the amplified C-93 allele (SEQ ID NO. 6) and amplified T-93 allele (SEQ ID NO. 7). In the semi-nested amplification reaction dTTP was replaced by dUTP. The 3' end of the forward primer was designed so that the first U incorporated downstream of the forward primer was at, or distal to, the polymorphic site in codon 93. Following amplification using P^{32} end labelled forward primer, glycosylase mediated cleavage of the amplified product was performed. Cleavage products were resolved by denaturing gel electrophoresis (20% polyacrylamide) and visualised by autoradiography. The C-93 allele was detected as a 32 n fragment (SEQ ID NO. 8)and the T-93 allele as a 27 n fragment (SEQ ID NO. 9).

The common 14 base pair insertion / deletion polymorphism in exon 8 of the HLA-G gene is referred to as I/D-E8 herein (also known as where I-E8 is one allele of the polymorphism and D-E8 is the other allele of the polymorphism. Genotyping of the HLA-G exon 8 deletion polymorphism was performed by amplifying a short section flanking the deletion location in exon 8. This was achieved using the polymerase chain reaction with primers designed to hybridise to known DNA sequence in exon 8. The forward primer was 5'-TGTGAAACAGCTGCCCTGTGT-3' (SEQ ID NO. 10) and the reverse primer was 5'-AAGGAATGCAGTTCAGCATGA-3' (SEQ ID NO. 11).

The polymerase chain reaction was carried out in a total volume of 25µl, with 100ng genomic DNA, 50ng of each primer, 0.2mM of each deoxyribonucleoside triphosphate (dATP, dCTP, dGTP and dTTP) 50mM KCl, 10mM Tris-HCl, pH 9.0 at 25°C, 0.1% Triton X-100, 0.5mM MgCl and 0.5U of Taq Polymerase. Reaction mixtures were covered with an equal volume of mineral oil and amplification was carried out using the "hot start" technique in a thermal cycler. The conditions for amplification involved denaturation at 94°C for 5 min followed by addition of Taq Polymerase. Thirty cycles were then performed: 94°C for 1 min, 54°C for 1 min, 72°C for 1 min and finally a 10 min extension at 72°C. The I/D exon 8 polymorphism was genotyped by size separation of the PCR products on a 10% non denaturing polyacrylamide gel and visualised by staining with ethidium bromide, the I-E8 insertion allele giving rise to a 151 bp product (SEQ ID NO. 12) and the D-E8 deletion allele giving rise to a 137 bp product (SEQ ID NO. 13).

Allele specific genotyping. In order to gain more information from the transmission of HLA-G polymorphisms in the second phase of the work, allele specific genotyping was performed. In the majority of cases. maternal and paternal C/T-93 and I/D-E8 HLA-G haplotypes could be directly assigned. In cases where all members of a trio were heterozygous for either C/T-93 or I/D-E8 polymorphisms, allele specific amplification was performed in order to assign haplotypes. This was achieved using allele

specific primers which allowed selective amplification of the I-E8 or D-E8 allele. Following allele specific amplification, the C/T-93 polymorphism was then genotyped using the GMPD assay described above. Conditions for amplification of the I-E8 allele were 30 cycles at 94°C for 45s, 64°C for 45s, 72°C for 60s using 5'-TACTCCCGAGTCTCCGGGTCTG-3' (SEQ ID NO. 1) as the forward primer and 5'-CAAAGGGAAGGCATGAACAAATCTTG-3' (SEQ ID NO. 14) as the reverse primer. Conditions for amplification of the D-E8 allele were 30 cycles at 94°C for 45s, 56°C for 45 s, 72°C for 60s using 5'-TACTCCCGAGTCTCCGGGTCTG-3' (SEQ ID NO. 1) as the forward primer and 5'-GTTCTTGAAGTCACAAAGGGACTTG -3' (SEQ ID NO. 15) as the reverse primer. Such allele specific amplification gives rise to four possible haplotypes, namely I-E8 and C-93 haplotype (SEQ ID NO. 16), I-E8 and T-93 haplotype (SEQ ID NO. 17), D-E8 and C-93 haplotype (SEQ ID NO. 18) and D-E8 and T-93 haplotype (SEQ ID NO. 19).

All individuals in both sets of trios were genotyped for some or all of these polymorphisms (table 1 and 2), transmitted and non-transmitted alleles were assigned and haplotypes were constructed. All individuals were genotyped for the C/T-93 and I/D-E8 polymorphism. Comparative statistical analysis was performed. In the second phase of the work more elaborate analysis was performed. Comparison of allele and haplotype frequencies and genotype distribution for the polymorphisms between and within the sample cohorts, and was performed using chi-squared contingency table analysis and/or log linear model analysis and/or transmission disequilibrium testing.

In cases where all members of a trio were heterozygous for C/T-93 or I/D-E8 polymorphisms, allele specific amplification was performed to determine haplotypes and thus assign transmitted and non-transmitted alleles. This was achieved using primers which allowed specific amplification of a section of the HLA-G gene from either the insertion, or, deletion in exon 8 to a site 5' of codon 93. The C/T-93 polymorphism was then genotyped in the specifically amplified allele. Using this approach transmitted and nontransmitted alleles to offspring were assigned.

Results

All of the genetic studies to date apart from the one concerning the HLA-G deletion polymorphism have examined the genotype of the pre-eclamptic mother. We took the view that foetal HLA-G is the most likely candidate gene for pre-eclampsia. Pre-eclampsia trios where the offspring was the offspring of the primagravida pre-eclampsia pregnancy and a control group of offspring of normal primagravida pregnancies were studied. 54 pre-eclamptic offspring and 48 control offspring were included in the investigation.

Genetic analysis of the HLA-G C/T-93 polymorphism in the pre-eclamptic offspring group revealed homozygosity for the C-93 allele in 7 cases (13%),homozygosity for the T-93 allele in 3 cases (5.6%) and heterozygosity in 44 cases (81.4%). In comparison, control offspring showed homozygosity for the C-93 allele in 18 cases (37.5%), homozygosity for the T-93 allele in 8 cases (16.6%) and heterozygosity in 22 cases (45.8%).

The frequencies of the C-93 and T-93 allele in the pre-eclamptic offspring were 0.537 and 0.463 respectively. In comparison, the frequencies in the control offspring were 0.604 and 0.396 respectively. The expected frequency distribution of the C-93 and T-93 alleles can be estimated with the formula p2 + 2pq + q2 = 1. With the allelic frequencies of p=0.537 and q=0.463 in the pre-eclamptic offspring group, the expected distribution of genotypes should be C-93/C-93 = 0.288, C-93/T-93 = 0.502 T-93/T-93 = 0.214. With the allelic frequencies of p=0.604 and q=0.396 in the control offspring group, the expected distribution of genotypes should be C-93/C-93 - 0.36, C-93/T-93 = 0.478, T-93/T-93 = 0.156. In comparison with the control group the distribution of genotypes in the pre-eclamptic offspring group is significantly different (Chi-square - 11.01, $p<0.001$, table 1).

It has been reported that no significant association was observed between the HLA-G exon 8 deletion polymorphism and pre-eclamptic offspring (Humphrey et al., 1995). In this work we also genotyped the pre-eclamptic and control offspring for the HLA-G deletion polymorphism. Genetic analysis of the HLA-G deletion polymorphism in the pre-eclamptic (n=51) and control offspring (n=55) groups revealed homozygosity for the normal allele in 12 cases (23.5%) and 13 cases (23.6%) respectively, homozygosity for the deletion allele in 8 cases (15.7%) and 12 cases (21.8%) respectively, and heterozygosity in 31 cases (60.8%) and 30 cases (54.5%) respectively.

The frequencies of the normal and deletion allele in the pre-eclamptic offspring were 0.539 and 0.46 respectively. In comparison, the frequencies in the control offspring were 0.509 and 0.491 respectively. In the pre-eclamptic offspring group, the expected distribution of genotypes should be normal allele/normal allele = 0.291, normal allele/deletion allele = 0.497, deletion allele/deletion allele = 0.212. The expected distribution of genotypes in the control offspring group should be normal allele/normal allele = 0.259, normal allele/deletion allele = 0.500, deletion allele/deletion allele = 0.241. In comparison with the control group the distribution of genotypes in the pre-eclamptic offspring group is not significantly different (Chi-square = 0.69, $p<0.30$, table 1).

The parents of the pre-eclamptic and control offspring were genotyped for the HLA-G C/T-93 genotype and the genotypes were analysed in conjunction with the offspring genotypes. In this analysis we scored the number of cases where the offspring had inherited a paternal C/T-93 allele that was not present in the maternal genotype. For the pre-eclamptic offspring, 41% of cases had a paternal allele of the C/T-93

genotype that was not present in the maternal genotype. By contrast, for the control offspring, 28% of cases had a paternal C/T-93 allele not represented in the maternal genotype.

Discussion

In this investigation, two common polymorphisms in the HLA-G gene in the offspring of pre-eclamptic and normal mothers were examined for association with pre-eclampsia. The two offspring groups are from Southern Ireland and are from the same ethnic background.

This is the first report determining association between pre-eclampsia in the mother and the foetal HLA-G genotype. Our results show a strong association between pre-eclampsia in the mother and heterozygosity for the C/T-93 polymorphism in offspring. This indicates that transmission of HLA-G alleles to offspring is different in normal offspring than in pre-eclampsia offspring. The result indicates that screening for susceptibility to pre-eclampsia can be achieved by genotyping of HLA-G in the mother and partner. Furthermore, since pre-eclampsia is associated with intrauterine growth retardation and miscarriage, it is likely that screening for susceptibility to intrauterine growth retardation, miscarriage and miscarriage-related infertility may also be achieved by HLA-G genotyping in the potential parents.

Following these results and to lend further support to this key finding, we expanded the number of subjects analysed to include additional normal primagravida trios where the mother had no history of pregnancy related problems, pre-eclampsia primagravida trios, families (mother, father, first and second offspring) where the mother had two or more successful normal pregnancies in the absence of pregnancy related disorders including pre-eclampsia and miscarriage, families (mother, father, first and second offspring) where the mother had pre-eclampsia in the first pregnancy and a normal second pregnancy in the absence of pregnancy related disorders including pre-eclampsia and miscarriage. As pre-eclampsia has been shown to be associated with miscarriage, we also included a cohort of recurrent miscarriage couples. The C/T-93 and I/D-E8 polymorphisms were genotyped in all individuals (Table 2 and Table 6), haplotypes were assigned and more elaborate statistics were applied to support our initial finding.

Linkage of HLA-G to pregnancy success in normal primagravidas

For transmission analysis of individual polymorphisms, we applied the log linear model and the transmission disequilibrium test. We also examined other polymorphisms in the HLA-G gene. The frequency of the A/T-31, A/T-107 and C/A-110 polymorphisms in the sample cohorts was 1.0/0.0), 0.95/0.05 and 0.94/0.06 respectively. For the analysis, we still utilised the commonly occurring C/T-93 and I/D-E8 polymorphisms. Using the allele specific amplification approach we assigned transmitted and nontransmitted alleles in the trios (Table 3). Since maternal genotype, foetal genotype (and specifically the paternal origin of foetal alleles) could potentially influence pregnancy outcome, a number of different

comparisons were made using this data. The log linear model of Weinberg et al. 1998 allows for causal scenarios in which the foetal genotype, parental genotypes or combinations thereof are directly relevant to risk. Maximum likelihood log-linear models of case-parent triad data were fitted. The log-linear model predicts the expected numbers of the 16 possible family types observed (for a bi-allelic marker), allowing for several "genetic risk factors" i.e. (a) whether the offspring carried one or more copies of an allele, (b) whether the mother carried one or more copies of an allele (c) a maternal origin effect and (d) a paternal origin effect (Table 4). The analysis was stratified on parental mating type assuming Hardy-Weinberg equilibrium, this corrects for the number of a particular allele that is found among the four parental alleles in a particular mating type, which has an obvious but uninteresting effect on allele distributions among the offspring. The advantage of this framework is that nested models of differing complexity are validly compared, starting with simple allelic effects and adding extra factors. We use a four-factor model, and then a stepwise reduced model which has removed the less significant factors automatically, thus providing a simpler model that accounts for important departures from expectations.

Fitting a four-parameter model to the data (Table 4) was significant for both the C-93 allele ($p=0.006$) and especially significant for the I-E8 allele ($p=0.0001$). Stepwise elimination of parameters which are not significant revealed that most of this can be accounted for by two effects: an effect due to foetal HLA-G alleles and, most strongly, by a parental origin effect for each allele (Table 4). Thus, both the C-93 and I-E8 alleles are significantly under-represented among offspring, and where they do occur, tend to be of paternal and maternal origin respectively. The maternal alleles in themselves are not significantly biased once these other effects are allowed for. The I-E8 allele is over 4 times more likely to be of maternal origin than expected (95% confidence interval 2.2-9.8).

When the transmission frequencies of maternal and paternal alleles to offspring were compared using chi-squared contingency table analysis, highly significant differences were observed for the C/T-93 and I/D-E8 alleles ($p\text{-}_1=0.009$ and $p\text{-}_1=0.000001$ respectively, Table 5, Table 3). This reflected a deficit in transmission of maternal C-93, D-E8 and paternal T-93, I-E8 alleles (Table 3). A significant difference was also observed between maternal and paternal non-transmitted I/D-E8 alleles showing that the maternal genotype plays a role in pregnancy outcome.

We verified that the highly significant findings from log-linear modelling by simpler comparisons. The transmission disequilibrium test transmission disequilibrium test assesses whether assess whether transmission of maternal and paternal alleles from heterozygous parents to offspring differed from the null expectation (of 50:50) and is valid even when Hardy-Weinberg equilibrium is violated by unusual population structure. When the transmission disequilibrium test was applied, the transmission of the C/T-93 and I/D-E8 alleles to offspring did not differ from the null expectation (Table 5). However, significant

deviations from the null expectation were observed when maternal and paternal transmission frequencies were analysed independently of each other.

Transmission of the maternal T-93 (chi-squared $_{\text{transmission disequilibrium test}}$, $p=0.032$) and I-E8 (chi-squared $_{\text{transmission disequilibrium test}}$, $p=0.0005$) alleles and also the paternal D-E8 allele (chi-squared transmission disequilibrium test, $p=0.01$) to offspring was markedly more frequent than expected (Table 5). There was an excess transmission of the maternal T-93 allele from heterozygous mothers to offspring. Specifically, thirty out of forty four offspring inherited the maternal T-93 allele from C/T-93 heterozygote mothers and forty out of fifty two offspring inherited the maternal I-E8 allele from I/D-E8 heterozygote mothers(Table 3). In addition, a contrasting excess transmission of the paternal D-E8 allele from heterozygote fathers to offspring (thirty one out of forty three cases) was observed (Table 3). These findings closely match the findings of the log-linear model.

We examined the data to determine if the transmission distortion could be accounted for by transmission to female or male offspring alone. However, there was no evidence that this was the case since of the forty out of fifty two offspring inheriting the maternal I-E8 allele from heterozygote mothers, 19 were females and 21 were males. Similarly, of the thirty one out of forty three offspring inheriting the paternal D-E8 allele from heterozygote fathers, 15 were females and 16 were males.

The primagravida mothers investigated here differed significantly from Hardy-Weinberg expectations ($p_1=0.006$) for I/D-E8 genotype frequencies (observed genotype frequencies: I/I; 17, I/D; 58, D/D; 15, expected I/I; 24, I/D; 45, D/D; 21). The transmission disequilibrium test results shows a significant effect without assuming Hardy-Weinberg equilibrium and thus support the log linear model results which was calculated assuming Hardy-Weinberg equilibrium.

Assignment of alleles transmitted and non transmitted to offspring was determined for five independent HLA-G polymorphisms permitting haplotype construction, and comparison of transmitted and non transmitted haplotypes. Thirteen haplotypes were observed (Table 6). Four of these, a-a-a-a-b, a-b-a-a-a a-a-a-a-a and a-b-a-a-b, were relatively common. Differences between the frequencies of maternal and paternal transmission were apparent for all four common haplotypes indicating that the distortion of HLA-G allele transmission to primagravida offspring could still be accounted for by biases in transmission of the C/T-93 and I/D-E8 polymorphisms. We then constructed haplotypes for the C/T 93 and I/D-E8 polymorphisms alone for comparison purposes.

The C-D and T-I haplotype were most common (Table 7). The strength of linkage disequilibrium between the two markers is indicated by the high frequency of the C-D haplotype, for which the disequilibrium, expressed as a proportion of the maximum disequilibrium (D/Dmax), is 0.344. Comparison of maternally

and paternally transmitted haplotypes to offspring using chi-squared contingency table analysis, revealed a highly significant difference between maternally and paternally transmitted haplotypes to offspring (p_3=0.000003) reflecting a deficit in transmission of both maternal haplotypes bearing the D-E8 allele (C-D and T-D) and both paternal haplotypes bearing the I-E8 allele (C-I and T-I) (Table 7). A significant difference was also observed between maternally and paternally non-transmitted haplotypes to offspring (p_3=0.028) showing that the maternal HLA-G genotype plays a role in pregnancy outcome.

Maternally and paternally transmitted haplotypes to individual offspring are shown in Table 8. The maternally transmitted T-I haplotype and the paternally transmitted C-D haplotype combination occurs in twenty one (34%) of the control offspring. By contrast the possible alternative combination (maternally transmitted C-D and paternally transmitted T-I) does not occur in any of the control offspring even though twenty two of the matings have this possibility.

In the primagravida offspring, homozygosity did not deviate from Hardy-Weinberg expectations. Comparison of the observed and expected number of homozygotes vs. heterozygotes within the offspring did not reveal any significant differences (p_1 = 0.256 for C/T-93 and p_1 = 0.82) showing that selection against homozygotes does not occur in primagravidas.

The significant distortion observed for transmission of HLA-G alleles to primagravida offspring provides evidence for maternal and paternal allele specific HLA-G based selection of foetuses in normal primagravidas. The selection observed was most pronounced for the HLA-G I/D-E8 polymorphism. The log linear model and transmission disequilibrium test analysis shows strong selection for the maternal I-E8 and paternal D-E8 allele in offspring. The selection effect is most dramatic for foetal combinations of the I/D-E8 alleles. In the total sample, thirty seven of the eighty four offspring have a maternal I-E8 paternal D-E8 allele combination. By contrast, there are only five offspring with the alternative maternal D-E8 paternal I-E8 allele combination (calculated from Table 8). These results show that maternal D-E8 paternal I-E8 foetuses are subject to significantly increased postzygotic prenatal loss and identify HLA-G as a key gene influencing this process. The deficiency of maternal D-E8 paternal I-E8 offspring is approximately 29% and closely matches postzygotic prenatal loss in prospective mothers which has been estimated to occur at a frequency of about 31% and in more than 20% of these cases, such loss occurs very early in pregnancy and is clinically unrecognisable.

The dichotomous effect whereby the maternal HLA-G I-E8 allele seemingly imparts a protective effect to the foetus while the equivalent paternal allele is detrimental is somewhat suggestive of genomic imprinting, however reports to date indicates that imprinting does not occur at the HLA-G locus.

It is not clear why there is an excess of heterozygote mother for the I/D-E8 genotype. The excess of heterozygote mother for the I/D-E8 genotype the population of mothers investigated have been selected on the basis of normal pregnancy outcome and as such represent a select group of the female population since the whole female population would include several other categories of females including infertile women (10-15% incidence), women that had a miscarriage (10-15% incidence) or pre-eclampsia (5-10% incidence).

In the primagravida offspring, homozygosity did not deviate from Hardy-Weinberg expectations. Comparison of the observed and expected number of homozygotes vs. heterozygotes within the offspring did not reveal any significant differences ($p_1 = 0.45$ for C/T-93 and $p_1 = 0.81$ I/D-E8) indicating that selection against homozygotes does not occur in primagravidas. The preferential transmission of a maternal I-E8 and paternal D-E8 HLA-G alleles to offspring might be expected to result in increased heterozygosity in the offspring. However, as the selection of offspring appears to be for maternal I-E8 and paternal D-E8 HLA-G allele combinations and against paternal I-E8 and maternal D-E8 allele combinations, the excess of the former heterozygote will be balanced by the deficiency of the latter.

As linkage disequilibrium occurs across the HLA locus and the results are proof that HLA-G and/or a HLA-G linked gene, cause the selection effects observed here. The C/T-93 polymorphism is a silent mutation while the I/D-E8 polymorphism occurs in the 3' untranslated region (UTR) of the gene. These polymorphisms have been considered innocuous. However, the evidence indicates that the deletion polymorphism has a functional effect on the HLA-G gene. The 14bp sequence of I/D-E8 polymorphism is largely conserved in primates and in the 3' UTR and/or in the last intron of HLA-B, C, J, A and E. 11 of the 14bp of the polymorphism is repeated in intron seven of the HLA-G gene. The core sequence "atttgt" is repeated one or more times in the 3' UTR of all class I genes but is absent in coding sequences. Examination of the secondary structure around the I/D-E8 polymorphism using the mfold programme (Zuker, 1994) shows that the 14n sequence is involved in a region of the 3'UTR having extensive secondary structure and that the secondary structure is altered depending on the presence or absence of the 14n sequence (data not shown). Thus the presence or absence of the polymorphism may affect the stability and/or alternative splicing of HLA-G mRNA through formation of alternative secondary structures.

Examination of human preimpantation blastocysts showed that only 40% of the blastocysts expressed HLA-G and such expression was associated with an increased cleavage rate by comparison with embryos lacking the HLA-G transcript. Thus, polymorphisms affecting expression of HLA-G are likely to influence the rate of postzygotic prenatal loss by altering the cleavage rate in the embryo.

Taken together, the results provide evidence that different HLA-G alleles and/or combinations thereof and/or variations in DNA in linkage disequilibrium with HLA-G in the foetus and/or one or both parents of the foetus are responsible for postzygotic prenatal loss which may manifest as miscarriage or undetectable early miscarriage which would manifest as unexplained infertility.

Identification of HLA-G as the Pre-eclampsia gene

A cohort of pre-eclampsia primagravida trios (mother, father and first offspring) were identified in maternity hospitals, sampled and genotyped for the following polymorphisms in the HLA-G gene: C/T at codon 93 (C/T-93) (Table 9), A/T at codon 107 (A/T 107), C/A at codon 110 (C/A 110), and the insertion / deletion polymorphism in the non-translated region of the gene in exon 8 (I/D-E8) (Table 9). Alleles transmitted and non transmitted to offspring were assigned (Table 10 and Table 11).

HLA-G genotypes and haplotypes in pre-eclampsia trios were examined independently using transmission segregation analysis. pre-eclampsia trios were also compared to the cohort of control primagravida trios. A significant difference in C/T-93 allele frequency was observed between control and pre-eclampsia mothers (p_1= 0.03, Table 12). A significant difference was also observed for the allele frequency of the I/D-E8 polymorphism between control and pre-eclampsia fathers (p_1= 0.02, Table 12). The frequency of the 93-E8 haplotypes differed significantly between control and pre-eclampsia mothers (p_3=0.03), control and pre-eclampsia fathers (p_3=0.008, Table 12) and also between control and pre-eclampsia offspring (p_3=0.03, Table 12).

The distribution of C/T-93 genotypes differed markedly between control and pre-eclampsia trios (Table 12), with a highly significant difference being observed between control and pre-eclampsia offspring (p_2=0.001), between control and pre-eclampsia fathers (p_2=0.02), and also between control and pre-eclampsia mothers (p_2= 0.05). These differences reflected a significant excess of C/T-93 heterozygotes over Hardy-Weinberg equilibrium expectations in both pre-eclampsia offspring (p_1=0.0002) and pre-eclampsia fathers (p_1 =0.021). A significant excess of I/D-E8 heterozygotes over Hardy-Weinberg expectations was also observed in control mothers, pre-eclampsia offspring and in pre-eclampsia fathers (Table 12).

Analysis of HLA-G haplotype sharing between offspring and mothers was also performed. No significant difference between pre-eclamptic cases and controls was observed for foetal-maternal sharing of HLA-G alleles or for sharing of the paternally transmitted HLA-G allele. There was no significant difference observed for offspring sex between controls and pre-eclampsia cases.

Comparison of the frequency of maternally and paternally transmitted alleles and haplotypes to control and pre-eclampsia offspring revealed significant differences (Table 12). In particular, these differences showed an excess of the maternally inherited T-93/I-E8 haplotype, and paternally inherited C-93/D-E8 haplotype and a deficiency of the maternally inherited C-93/D-E8 haplotype, and paternally inherited T-93/I-E8 haplotype in control offspring by comparison with pre-eclampsia offspring (Table 10). Furthermore, there was a significant difference between non-transmitted maternal I/D-E8 alleles and 93-E8 haplotypes showing that the maternal non-transmitted alleles are associated with pregnancy outcome.

Only twelve out of fifty two control offspring inherited the maternal D-E8 allele from heterozygous (I/D-E8) mothers (Table 3). By contrast, the maternal D-E8 allele was transmitted to twenty one of thirty six pre-eclampsia offspring (Table 11). Taken together, these findings show a significant deficit of maternal D-E8 transmission to control offspring and a contrasting excess of maternal D-E8 transmission to pre-eclampsia offspring.

Further analysis within control and pre-eclampsia trios was achieved by comparison of maternally and paternally transmitted alleles and haplotypes to the offspring. In control offspring a highly significant difference was observed between transmission of maternal and paternal C/T-93 alleles (Table 5, p_1=0.009, calculated from Table 3). This reflected a deficit of maternal C-93 and paternal T-93 transmitted alleles (Table 3). A highly significant difference was also observed between transmission of maternal and paternal I/D-E8 alleles (Table 4, p_1=0.000001, calculated from Table 3), showing a deficit of transmission of maternal D-E8 and paternal I-E8 alleles to the offspring (Table 3). A significant, but contrasting difference between transmission of maternal and paternal alleles was present in pre-eclampsia offspring where an excess of transmission of maternal D-E8 and paternal I-E8 alleles, maternal C-93 and paternal T-93 was observed (Table 13, Table 10). A significant difference was present between maternally and paternally transmitted haplotypes in both control and pre-eclampsia offspring (p_3=0.000003, Table 5 and p_3=0.005 respectively) showing a deficit in transmission of maternal C-93/D-E8 haplotypes and paternal T-93/I-E8 haplotypes to the control offspring and an excess in transmission of maternal C-93/D-E8 haplotypes and paternal T-93/I-E8 haplotypes to the pre-eclampsia offspring (Table 10).

Maternally and paternally transmitted haplotypes to individual offspring are shown in Table 14. The maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combination occurs in twenty two (35%) of the pre-eclampsia offspring but does not occur in any of the control offspring. By contrast, the paternal C-93/D-E8, maternal T-93/I-E8 haplotype combination occurs in both control and pre-eclampsia offspring but is in excess in the control offspring. This finding

provides evidence that combinations of HLA-G alleles / haplotypes in the foetus are causative of pre-eclampsia in the mother.

Taken together, the results provide evidence that different foetal HLA-G alleles and/or combinations thereof and/or variations in DNA in linkage disequilibrium with HLA-G in the foetus and/or one or both parents of the foetus are responsible for pre-eclampsia

Association of HLA-G with Recurrent Miscarriage

A cohort of couples where the mother had three or more consecutive miscarriages were identified, sampled and genotyped for the C/T-93 and I/D-E8 polymorphisms in the HLA-G gene and 93-I-E8 haplotypes were assigned. RSA mothers and RSA fathers were compared to the cohort of control and pre-eclampsia primagravida trios for C/T-93 and I/D-E8 allele frequency, C/T-93 and I/D-E8 genotype distribution and 93-E8 haplotype frequency. The genotypes and haplotypes of the couples are shown in Table 16.

A significant difference in C/T-93 allele frequency was observed between control and pre-eclampsia mothers (p_1= 0.03) and control and RSA mothers (p_1= 0.002) but not between pre-eclampsia and RSA mothers. The frequency of the 93-E8 haplotypes differed significantly between control and pre-eclampsia mothers (p_3=0.03), control and RSA mothers (p_3=0.01), but not between pre-eclampsia and RSA mothers.

The 93-E8 haplotypes of female and male mating partners were constructed. 50% of couples have the possibility of producing foetuses with the maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combination. This compares with a 24% possibility in control couples and a 44% possibility in pre-eclampsia couples. The possibility of producing foetuses with the maternally transmitted D-E8 allele and the paternally transmitted I-E8 allele is 46% for control couples, 70% for pre-eclampsia couples and 85% for recurrent miscarriage couples. Maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combinations in foetuses are only found in pre-eclampsia offspring. Recurrent miscarriage couple no. 15 must produce such a foetus. Taken together, the results provide evidence that foetal genotype associated with pre-eclampsia are also associated with miscarriage. Finally, mating couples where the female is homozygous for the T-93/I-E8 haplotype and the male has the C-93/D-E8 and T-93/I-E8 haplotypes were found in seven of sixty three pre-eclampsia cases and were absent in controls. Two of twenty of the recurrent miscarriage mating couples (no. 10 and 13, Table 17) also had the same haplotype combinations. This provides evidence that partners where the female is homozygous for the T-93/I-E8 haplotype and the male has the C-93/D-E8 and T-93/I-E8 haplotypes are susceptible to pre-eclampsia and/or miscarriage. Taken together, the results

provide evidence that miscarriage and pre-eclampsia are closely related and that miscarriage is a severe expression of PE. One offspring of T-93/I-E8 and C-93/D-E8 haplotype combination in control trios was found where the mother was homozygous for T-93/I-E8 and father had the C-93/D-E8 haplotype and the C-93/I-E8 haplotype. One offspring of T-93/I-E8 and T-93/I-E8 haplotype combination in control trios was also found where the mother was homozygous for T-93/I-E8 and father had the T-93/I-E8 haplotype and the C-93/I-E8 haplotype. This result shows that the non-transmitted male haplotype has a major influence on pregnancy outcome and indicates that sperm/semen contains a factor which influences susceptibility to pre-eclampsia and miscarriage.

Taken together, the results provide evidence that different HLA-G alleles and/or combinations thereof and/or variations in DNA in linkage disequilibrium with HLA-G in the foetus and/or one or both parents of the foetus are responsible for miscarriage.

Induction of tolerance to HLA-G pre-eclampsia/miscarriage haplotypes in first pregnancy

The results show that maternally transmitted D-E8 allele and the paternally transmitted I-E8 to offspring are linked to pregnancy outcome and that the maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combination cause pre-eclampsia and miscarriage in primagravidas. We analysed HLA-G transmission in fifty three couples that have had two successful pregnancies without a history of miscarriage or PE.

The possibility of producing foetuses with the maternally transmitted D-E8 allele and the paternally transmitted I-E8 allele was 15% for this cohort of normal couples. The results thus show a clear correlation between successful pregnancy outcome and the probability of possibilities of producing foetuses with the maternally transmitted D-E8 and the paternally transmitted I-E8 HLA-G alleles. Recurrent miscarriage - 85%, pre-eclampsia - 70%, first pregnancy normal - 46%, first and second pregnancy normal 15%.

The maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combination cause pre-eclampsia and miscarriage in primagravidas. We determined if primagravida normal pregnancy induced tolerance to the foetal pre-eclampsia / miscarriage haplotype combinations when they occur in pregnancy two. There were no first offspring detected bearing the maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combination. There were five second offspring bearing the maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combination (Table 18). We also determined if primagravida pre-eclampsia pregnancy induced tolerance to the foetal pre-eclampsia / miscarriage haplotype combinations when they occur in pregnancy two. We analysed nine families where the mother suffered pre-eclampsia in

her first pregnancy and had a normal second pregnancy. There were three second offspring bearing the maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype combination in the absence of pre-eclampsia even though the same combination caused pre-eclampsia in the first pregnancy (Table 19).

This proves that tolerance to the paternal antigens in the foetus is induced in the first pregnancy. More specifically, tolerance to the problematic maternally transmitted C-93/D-E8 haplotype and the paternally transmitted T-93/I-E8 haplotype was induced in first pregnancy. Thus exposure to HLA-G alleles and/ or combinations thereof and/or paternal antigens presented to the maternal immune system by HLA-G in the first pregnancy induces tolerance to the pre-eclampsia / miscarriage haplotype combination so that these problematic haplotypes can occur in the second pregnancy without associated pre-eclampsia or miscarriage.

The genetic linkage, association and correlation approaches used in the large number of subject cohorts provides proof that HLA-G is a susceptibility gene for normal pregnancy, pre-eclampsia and miscarriage. As pre-eclampsia is associated with intra uterine growth retardation, the HLA-G gene is also a susceptibility gene for intra uterine growth retardation. As miscarriage frequently occurs so early that it is not detected, the HLA-G is also a susceptibility gene for miscarriage related unexplained infertility. Exposure to foetal antigens including HLA-G in the first pregnancy has been shown to induce tolerance to antigens that are problematic in first pregnancy and thus provide a means for potential treatment of pre-eclampsia, miscarriage, intrauterine growth retardation, miscarriage related infertility and autoimmune disease and provide a means to induce tolerance to foreign antigens for purposes such as transplantation of foreign tissue.

The HLA-G I/D-E8 polymorphism has been investigated previously in pre-eclampsia and no detectable relationship was observed between susceptibility to pre-eclampsia and HLA-G (24). This result is consistent with the results reported here in that an HLA-G effect is not seen when the I/D-E8 polymorphism is independently analysed by association studies alone.

The results presented here show that genetic screening of maternal and/or paternal and/or foetal DNA is of value for predictive testing of susceptibility to pre-eclampsia, eclampsia, intrauterine growth retardation, miscarriage and miscarriage-related infertility. Furthermore, transmission of HLA-G alleles to offspring in normal pregnancy differs from the normal expectation. Therefore, the results presented here show that genetic screening of maternal and/or paternal foetal DNA is of value for predictive testing of susceptibility to normal pregnancy.

Preferably, foetal nucleic acid is isolated from any material containing nucleic acid of foetal origin in the mother such as amniotic fluid, maternal blood or chorionic villus. Furthermore, the results show that genetic screening of parents will also be of value for predictive testing of susceptibility to pre-eclampsia.

Although the function of HLA-G apart from its role in regulating NK cell activity and induction of IL-3 and IL-1 beta in PBMCs is as yet poorly understood, HLA-G is an excellent candidate for pre-eclampsia since HLA-G is considered to play a key role in foetal-maternal immune interactions. The C/T-93 HLA-G allele associated with pre-eclampsia is a silent polymorphism but its effect on HLA-G mRNA stability or splicing is unknown. It is likely that this polymorphism and/or variations linked to this polymorphism play a causative role in pre-eclampsia.

In this work, we have demonstrated a difference between pre-eclamptic and control offspring with respect to sharing of the paternal C/T-93 allele between the offspring and their mothers. This result indicates that pre-eclampsia may arise due to the presence of a HLA-G haplotype in the foetus that has not previously been encountered by the mother. Since HLA-G is in tight linkage disequilibrium with the HLA locus, it is likely that the paternal HLA-G itself and/or the presence of an extended paternal HLA haplotype in the foetus that has not previously been encountered by the mother causes pre-eclampsia. Furthermore, since HLA-G is in tight linkage disequilibrium with the HLA locus, determination of the extended paternal HLA haplotype segregating in the foetus and comparison of the haplotype with the maternal HLA haplotypes will allow diagnosis of susceptibility to pre-eclampsia.

While other associations have been reported between pre-eclampsia and the maternal genotype, the results reported here are much more consistent with epidemiological studies on pre-eclampsia. In particular, the association between a foetal HLA-G genotype is consistent with the observation that a) pre-eclampsia is more common in sisters than in the normal population, b) pre-eclampsia is discordant in identical twin mothers and c) pre-eclampsia can occur with a change of male partner. Pre-eclampsia is rare in second or later pregnancies indicating that initial exposure to functional HLA-G prevents pre-eclampsia. In addition, HLA-G is now known to induce synthesis IL-3 and IL-1 beta and down-regulate tumour necrosis factor-alpha production. These observations coupled with the results presented here indicates that HLA-G protein, IL-3 and/or IL-1 beta or inhibitors of tumour necrosis factor-alpha will be useful for treatment of intrauterine growth retardation, pre-eclampsia, miscarriage and miscarriage-related infertility.

The HLA-G genotype associated with pre-eclampsia and miscarriage is likely to have one of a small number of consequences:

i) it could result in reduced expression of HLA-G which would be reflected as decreased levels of cellular and/or soluble HLA-G (the HLA-G primary transcript is alternatively spliced to yield several

different mRNAs. One of these alternatively spliced forms includes intron 4. The open reading frame in this mRNA continues into intron 4, terminating 21 amino acids after the alpha3 domain - encoded by exon 4. Thus, the transmembrane region encoded by exon 5 and the cytoplasmic tail of HLA-G is excluded. The resultant protein is hence soluble). Thus measuring of cellular and/or soluble HLA-G levels and comparing these levels with the normal observed levels would allow one to diagnose susceptibility to pre-eclampsia and miscarriage;

ii) the HLA-G genotypes associated with pre-eclampsia may lead to variations in HLA-G mRNA and/or HLA-G protein which in turn could be detected by characterisation of HLA-G mRNAand/or protein. Thus, characterisation of HLA-G protein in pregnant females, foetuses and/or respective male mating partner would allow one to diagnose susceptibility to pre-eclampsia and miscarriage;

iii) expression of the HLA-G protein leads directly or indirectly to alterations in the levels of certain molecules such as IL-3, IL-1 beta and/or tumour necrosis factor alpha. The HLA-G genotypes associated with pre-eclampsia may result in changed expression of such molecules. Thus measuring of the levels of such molecules and comparing these levels with the normal observed levels would allow one to diagnose susceptibility to pre-eclampsia and miscarriage;

iv) The HLA-G genotypes associated with pre-eclampsia may result in decreased expression of HLA-G. This in turn would lead to increased lysis of trophoblasts by NK cells. Thus measuring of the levels of trophoblast specific marker and comparing these levels with the normal observed levels would allow one to diagnose susceptibility to pre-eclampsia and miscarriage.

The HLA-G variants associated with pre-eclampsia and miscarriage and normal pregnancy are likely to have one of a small number of consequences:

i) a variant could result in altered expression of HLA-G splice forms and levels thereof which would be reflected as altered levels of HLA-G splice forms including soluble HLA-G in the serum.. Thus measuring of size, levels and/or splice forms of HLA-G mRNA and/or protein including soluble HLA-G levels and comparing these levels with the normal observed levels would allow one to diagnose susceptibility to pre-eclampsia and miscarriage;

ii) the HLA-G variants associated with pre-eclampsia and miscarriage may result in variations in HLA-G protein which in turn could be detected by protein characterisation of cellular and/or soluble HLA-G. Thus characterisation of HLA-G protein in pregnant females, foetuses and/or respective male mating partner would allow one to diagnose susceptibility to pre-eclampsia and miscarriage;

iii) expression of the HLA-G protein leads directly or indirectly to alterations in the levels of certain molecules such as IL-3, IL-1 beta and/or tumour necrosis factor alpha. The HLA-G variants associated with pre-eclampsia may result in changed expression of such molecules. Thus measuring of the levels of such molecules and comparing these levels with the normal observed levels would allow one to diagnose susceptibility to pre-eclampsia and miscarriage;

iv) the HLA-G variants associated with pre-eclampsia and miscarriage may result in increased or decreased expression of paternal and/or maternal HLA-G. This in turn would lead to increased lysis of trophoblasts by NK cells and/or cytotoxic T cells. Thus measuring of the levels of trophoblast specific marker and comparing these levels with the normal observed levels would allow one to diagnose susceptibility to pre-eclampsia.

v) the HLA-G variants associated with pre-eclampsia and miscarriage may result in increased or decreased cell cleavage rates in the embryo. Thus measuring of cell cleavage rates in cells expressing one or more HLA-G variants and any combinations thereof would allow one to diagnose susceptibility to pre-eclampsia and miscarriage.

The results show that HLA-G polymorphism plays a major role in predisposition to normal, pre-eclampsia and miscarriage outcome in pregnancy and that haplotypic combinations and parent-of-origin effects mediate the influence of HLA-G polymorphism on these outcomes. The results show a strong association between foetal and paternal HLA-G genotypes and PE, and analysis of heterozygote v. homozygote mating outcomes indicate that transmission of HLA-G alleles to the pre-eclampsia offspring, but not to control offspring, is distorted. The results provide evidence for linkage of the maternal HLA-G I-E8 allele to normal pregnancy outcome in primagravidas and the observed deficit of maternal D-E8 allele and C-93/D-E8 haplotype transmission to control offspring indicates selection for foetuses on the basis of HLA-G genotype in primagravida normal pregnancies. The transmission distortion of the maternal D-E8 allele to the foetus indicates that the effect seen in normal primagravidas is mediated by the maternal allele acting primarily in the foetus. Thus, the maternal HLA-G imparts a protective effect to the foetus which enhances normal pregnancy outcome. This finding indicates that maternal selection of the HLA-G I-E8 and other protective HLA-G alleles occurs in normal pregnancy. By contrast, the maternal D-E8 allele was prevalent in heterozygous pre-eclampsia offspring, indicating that susceptibility to pre-eclampsia partly arises through the lack of a protective maternal HLA-G allele in the foetus. The chi-squared contingency table analysis agreed with the log linear model analysis in that the C-93 allele was over-represented in pre-eclampsia offspring and a bias towards maternal inheritance of I-E8 was present in controls. Furthermore, the log linear model showed that the foetal C-93 allele is under-represented in control offspring with a strong bias towards paternal inheritance of the allele. This indicates that the paternal C-93 allele also imparts a protective or alternatively does not introduce a problematic effect to the foetus which improves the prospect of a normal pregnancy outcome. These results are in good agreement with the findings observed when maternal and paternal haplotype combinations were constructed for individual control and pre-eclampsia offspring where more than one third of the pre-eclampsia cases had a maternal C-93/D-E8 paternal T-93/I-E8 haplotype combination that was absent in the controls. Taken together, the data indicate a strong association between both maternal and paternal HLA-G alleles acting through the foetus and normal pregnancy outcome and indicate that pre-eclampsia

arises through the absence of protective maternal and protective or problematic paternal HLA-G alleles in the foetus. Furthermore, considering that there are likely to be several HLA-G alleles with functional differences, and as more than one third of pre-eclampsia cases can be accounted for by a particular maternal / paternal haplotype combination, the results show that the magnitude of the effect of HLA-G in normal and pre-eclampsia pregnancies is large.

Alternatively, a protective foetal-maternal HLA-G allele is likely to arise through the transmission of a dominant maternal allele to the foetus which is recognised as self by the maternal immune system. A protective foetal-paternal allele is likely to arise through cross recognition of the paternal allele as self by the maternal immune system. A problematic foetal-paternal allele is likely to arise through cross recognition of the paternal allele as non- self by the maternal immune system. The results indicate maternal education of the lymphocyte repertoire for maternal HLA-G during and/or prior to pregnancy and for paternal HLA-G during pregnancy. The results also indicate certain paternal HLA-G alleles are compatible with the maternal immune system while others are less compatible. Combinations of less compatible/incompatible paternal HLA-G alleles with maternal alleles which do not protect against the paternal alleles are likely to cause susceptibility to pre-eclampsia and miscarriage.

The fact that second offspring of primagravida normal and pre-eclampsia mothers have the maternal C-93/D-E8 paternal T-93/I-E8 genotype in the absence of pre-eclampsia in the second pregnancy is evidence that maternal education for foetal-paternal antigens occurs during the first pregnancy and that this education is mediated by HLA-G. It is clear from this work that the polymorphisms analysed and/or closely linked polymorphisms in HLA-G or flanking HLA genes contribute directly to enhancing normal pregnancy outcome and to susceptibility to pre-eclampsia and miscarriage. One likely explanation may be that the polymorphisms reported here destabilise HLA-G mRNA and/or alter the splicing pattern and/or glycosylation pattern of HLA-G. The presence or absence of polymorphism is likely to effect the stability and/or alternative splicing of HLA-G mRNA. Thus a protective foetal-maternal HLA-G allele is likely to arise through the transmission of a maternal allele to the foetus which may or may not be expressed in the embryo. A protective foetal-paternal allele is likely to arise through the transmission of a paternal allele to the foetus which may or may not be expressed in the embryo.

At least twelve different haplotypes have been described for the HLA-G gene. Considering the link observed between HLA-G and recurrent miscarriage, it is likely that the combination of HLA-G alleles in the early foetus and/or the combination of the HLA-G alleles in the mother has serious effects on the outcome of implantation in general and is likely to account for cases of unexplained or idiopathic infertility as well as miscarriage. The previously reported link between pre-eclampsia and intra-uterine

growth retardation indicates that the latter is also likely to be linked to parent of origins effects of foetal HLA-G alleles and indicate that maternal HLA-G alleles also play a role in the foetal growth outcome.

HLA-G is capable of protecting otherwise susceptible target cells from natural killer cell mediated lysis through its interaction with inhibitory receptors on natural killer cells. HLA-G is also capable of stimulating an HLA-G restricted lymphocyte response. HLA-G molecules can serve as target molecules in lytic reactions with lymphocytes, and HLA-G is involved in education of the lymphocytic repertoire. Thus, pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation is likely to arise through a mechanism involving blood mononuclear cells such as natural killer cells and cytotoxic T lymphocytes whereby interaction between the female mating partner's T cells and foetal antigens is compromised by comparison with normal pregnancy. Thus, compromised interaction leading to the lack of tolerance leads to cell killing. Compromised interaction also can lead to lack of stimulation of cells expressing HLA-G molecules and/or lack of stimulation of cells interacting with cells expressing HLA-G molecules. The fact that the maternal C-93/D-E8 paternal T-93/I-E8 HLA-G genotype can occur in the second pregnancy of a primagravida pre-eclampsia case without pre-eclampsia indicates that education mediated by foetal HLA-G to foetal antigens occurs in the first pregnancy of such mothers which overcomes compromised interactions in second and subsequent pregnancies . The fact that a deficit of maternal C-93/D-E8 genotypes and an excess of T-93/I-E8 genotypes are transmitted to control offspring but not to pre-eclampsia offspring indicates that selection for foetuses that express antigens for which the mother is educated occurs in normal pregnancy. The fact that pre-eclampsia rarely occurs in a second pregnancy when the first pregnancy has been normal indicates that induction of education to foetal antigens mediated by HLA-G also occurs during and prior to the first pregnancy in normal mothers and that pre-eclampsia, miscarriage, miscarriage-related infertility and intra-uterine growth retardation arises from lack of education and/or inadequate induction of education to the foetal antigens in the female mating partner during and/or prior to pregnancy. Lack of and/or compromised induction of education to paternal antigens such as HLA-G in the foetus and/or a defective HLA-G interaction with natural killer cells could lead to lysis of trophoblasts and/or lack of stimulation of trophoblasts leading to reduced trophoblast function and/or lack of stimulation of cells interacting with trophoblasts. Thus, HLA-G linked conditions such as pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation are likely to arise through blood mononuclear cell mediated killing of accessible foetal tissues such as trophoblasts and/or lack of stimulation of trophoblastic cells because of compromised HLA-G interaction with blood mononuclear cells trophoblasts and/or lack of stimulation of blood mononuclear cells because of compromised HLA-G interaction with trophoblastic cells. Since major histocompatibility (MHC) molecules like HLA-G interact with blood mononuclear cells including cytotoxic T cells and natural killer cells, there is likely to be abnormal interaction between maternal blood mononuclear cells and foetal cells presenting MHC / MHC-antigen complexes and/or MHC / MHC-antigen complexes

secreted from foetal cells in pre-eclampsia, miscarriage and intra-uterine growth retardation by comparison with normal pregnancies. Thus, the blood mononuclear cell response and/or the trophoblast response to such an interaction is likely to be abnormal in the HLA-G associated disorders. In particular, the cytokine response produced as a result of such an interaction is likely to be abnormal by comparison with the normal situation.

Thus, diagnosis of susceptibility to pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation and prediction of pregnancy outcomes may be achieved by direct and indirect measurement of the education in the female mating partner to foetal antigens and/or direct and indirect measurement of the interaction between blood mononuclear cells and HLA-G and/or HLA-G expressing cells. Furthermore, direct and indirect measurement of the education in the female mating partner to foetal antigens and/or direct and indirect measurement of the natural killer cell activity in the female mating partner to HLA-G expressing cells and/or direct and indirect measurement of the interaction between blood mononuclear cells and HLA-G and/or HLA-G expressing cells offers a means to monitor the course of pregnancy.

Induction of education to foetal antigens in the female mating partner by treatment with HLA-G and/or peptides known to bind to HLA-G constitutes a therapeutic means for prevention and/or treatment of pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation and any other HLA-G related disorders.

The finding that combinations of HLA-G variants in the foetus are closely associated with pre-eclampsia and miscarriage coupled to the fact that HLA-G interacts with blood mononuclear cells offers a further means to prevent and/or treat pre-eclampsia miscarriage, miscarriage-related infertility and intrauterine growth retardation by inhibition and/or alteration of the interaction of HLA-G and/or HLA-G variants with blood mononuclear cells. This may be achieved by any one or combination of approaches including treatment with one or more molecules which recognise HLA-G and/or variants of HLA-G and/or one or more HLA-G receptors on blood mononuclear cells, and/or inactivation of the HLA-G gene and/or HLA-G gene variants and/or one or more HLA-G receptors on blood mononuclear cells. For example, this would be achieved by treatment with HLA-G specific and/or HLA-G receptor specific antibodies which interfere with HLA-G - blood mononuclear cell interaction and/or treatment with one or more enzymes which recognise and alter HLA-G and/or HLA-G receptors on blood mononuclear cells and/or treatment with one or more peptides which bind to HLA-G and/or HLA-G receptors on blood mononuclear cells. Alternatively, inhibition of the interaction of one or more HLA-G variants with blood mononuclear cells may be achieved by inactivating the HLA-G gene or HLA-G gene variant and/or one or more HLA-G receptors on blood mononuclear cells. This may be achieved through the use of one or more gene

inactivating approaches such as treatment with one or more nucleic acid antisense and/or ribozyme molecules which inhibit expression of the HLA-G gene and/or HLA-G gene variant and/or one or more HLA-G receptors on blood mononuclear cells. This may be also be achieved by inactivating the HLA-G gene in one or both partners of a mating couple somatically or in the germ line through the use of gene therapy approaches whereby inhibitory nucleic acid based molecules such as antisense, and/or ribozyme are introduced into an individual. This may be also be achieved by inactivating the HLA-G gene in one or both partners of a mating couple somatically or in the germ line through the introduction of all or part of a HLA-G gene in such a way that it recombines with the endogenous HLA-G in the cell and inactivates it. Alternatively, the HLA-G gene and/or variants of the HLA-G gene and/or any of it's receptors may be employed in gene therapy methods in order to increase the amount of expression products of such genes in an individual allowing compensation of any deficiency of HLA-G and/or it's receptors in an individual. Thus, alteration of the interaction of HLA-G and/or HLA-G variants with blood mononuclear cells may be achieved by introduction of one or more HLA-G gene variants into somatic cells and/or into the germline of one of both partners of a mating couple or into the fertilised egg or cells arising from the fertilised egg prior to implantation. This is of particular importance for increased fertility for animal breeding purposes. For example, introduction of one or more HLA-G gene variants into the germline of one of both partner of a mating couple or into the fertilised egg or cells arising from the fertilised egg where the HLA-G variant is compatible with the prospective mother offers a means to improve fertility and pregnancy outcome arising from any incompatibility between foetal HLA-G and maternal cells in the mother.

HLA-G binds a diverse but limited array of peptides in a manner similar to that found for classical class I molecules and it has been reported that HLA-G is expressed in the human thymus raising the possibility that maternal unresponsiveness to HLA-G expressing foetal tissues may be shaped in the thymus by central presentation of this MHC molecule on the medullary epithelium (Crisa *et al.* 1997) HLA-G is known to be capable of stimulating a HLA-G restricted cytotoxic T lymphocyte response and HLA-G molecules can serve as target molecules in lytic reaction with cytotoxic T lymphocytes and HLA-G expressed internally in vivo in transgenic animals is involved in education of the lymphocytic repertoire (Schmidt *et al.*, 1997). The invention shows that the induction of education to foetal antigens occurs during pregnancy and arises from exposure of the mother to foetal antigens during pregnancy. HLA-G allele combinations that were unacceptable in first pregnancy and/or were linked to pre-eclampsia were acceptable in second pregnancy without any associated pregnancy complications. Thus, induction of education to foetal antigens is likely to arise from a process involving HLA-G. Thus the invention offers a means of inducing education including tolerance to HLA-G and/or peptides bound to HLA-G in an individual through mimicking the exposure to foetal antigens that occurs during pregnancy. Thus treatment of an individual with HLA-G and or / peptides known to bind to HLA-G constitutes a means to

induce education in an individual to antigens. In particular, this offers a means to induce tolerance to antigens that cause susceptibility to pre-eclampsia, susceptibility to miscarriage, autoimmune disease and transplant rejection.

In normal pregnancy, direct and indirect alteration of the level and/or activity of molecules arising from the interaction of HLA-G expressing foetal cells with blood mononuclear cells such as lymphocytes and natural killer cells permit pregnancy to progress properly. In pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation and any other HLA-G related disorders, the alteration of the level and/or activity of molecules arising from the interaction of HLA-G and/or HLA-G expressing foetal cells with blood mononuclear cells such as lymphocytes and natural killer cells is likely to be compromised by comparison with that occurring during normal pregnancy. Thus, mimicry of the alteration of the level and/or activity of one or more molecules arising from the interaction of HLA-G and/or HLA-G expressing foetal cells with blood mononuclear cells in an individual constitutes a therapeutic means for prevention and/or treatment of pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation and any other HLA-G related disorders.

The deficit of maternal HLA-G C-93/D-E8 genotypes and the excess of T-93/I-E8 genotypes transmitted to control offspring but not to pre-eclampsia offspring implies selection of foetuses in normal pregnancy dependent on HLA-G genotype. For fertility purposes, and especially in vitro fertilisation and embryo transfer in animals, selection of one or both mating partners, sperm and/or egg donors and/or embryo recipients based on male and/or female HLA-G and/or HLA-G homologue genotypes and/or serotypes and/or activity associated with a successful normal first pregnancy with a specific mating partner offers a means to improve fertility and the success rate of in vitro fertilisation and embryo transfer in animals and improve pregnancy outcome.

Since HLA-G protects trophoblasts from blood mononuclear cell mediated killing, direct and indirect measurement of measurable substances which originate from trophoblast cell killing should allow diagnosis of susceptibility to pre-eclampsia, miscarriage, intra-uterine growth retardation , and monitoring of pregnancy for normal progress, and progress towards pre-eclampsia, miscarriage and intra-uterine growth retardation in humans and animals. More specifically, the interaction between MHC molecules such as HLA-G and blood mononuclear cells is known to directly and indirectly alter the synthesis and levels of several cytokines. Similarly, trophoblasts are known to synthesise and secrete several cytokines. In particular, the altered regulation of some of these cytokines would be expected to compromise the foetal - maternal immune interaction and could be manifest as pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or miscarriage and/or miscarriage-related infertility. For example, the interaction of HLA-G expressing cells with blood mononuclear cells increases the amount of interleukin-

3 (IL-3) and interleukin-1 beta (IL-1 beta) and decreases the amount of tumour necrosis factor-alpha (TNF-alpha) release from the blood mononuclear cells. Trophoblasts are known to produce the immunosuppressive cytokine interleukin 10 - a cytokine that potently inhibits alloresponses in mixed lymphocyte reactions. Trophoblasts are also known to produce interleukin 2, a cytokine that both protects the foetus and in involved in activation of maternal killer cells to protect against invading trophoblasts, interleukin 4 and its receptor, which play a role in regulation of umbilical blood flow mediated through the induction of cyclooxygenase-2, indicating a role for interleukin 4 in vascular tone and blood flow modulation during pregnancy, interleukin 6, which is likely to play a role in tissue remodelling associated with placentation.

Since the indications are that pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation arise through a HLA-G mediated mechanism, there are several obvious methods for screening for agents which can potentially be used as diagnostic indicators and therapeutic agents. Screening of gene expression profiles using DNA probe arrays allows identification of genes expressed in HLA-G expressing cells and in blood mononuclear cells and genes whose expression changes as a result of HLA-G interaction with blood mononuclear cells. Comparison of the gene expression profile in HLA-G expressing cells and/or blood mononuclear cells and/or HLA-G expressing cells interacting with blood mononuclear cells and/or in blood mononuclear cells interacting with HLA-G allows identification of agents which can potentially be used as diagnostic indicators and therapeutic agents for pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation.

HLA-G function and HLA-G expression can be measured. Thus screening for agents which alter the expression and/or function and/or which mimic the function of HLA-G provide a method for screening for potential pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation therapeutic agents.

The words "comprises/comprising" and the words "having/including" when used herein with reference to the present invention are used to specify the presence of stated features, integers, steps or components but does not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

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Table 1: Genotype and allele distribution of the HLA-G C/T-93 (C1488T) and I/D-E8 (exon deletion) polymorphisms in pre-eclamptic and control offsrping.

Polymorphism	genotype	control offspring	preeclamptic offspring	χ^2	p
	Aa	22	44		
HLA-G C/T-93 polymorphism	aa	8	3	6.11	<0.02
	AA	18	7	11.01	<0.001
	Aa	30	31		
HLA-G C/T-93 polymorphism	aa	12	8	0.69	0.3
	AA	13	12	0.06	<0.8

Table 2: Genotype and allele distribution of the HLA-G C/T-93 and I/D-E8 polymorphisms in primigravida trios.

		C/T-93			Frequency
	n	C/C (%)	C/T (%)	T/T (%)	C/T
Mothers	90	19(21.1)	50(55.6)	21(23.3)	0.49/0.51
Fathers	90	34(37.8)	39(43.3)	17(18.9)	0.59/0.41
Offspring	90	24(26.7)	41(45.5)	25(27.8)	0.49/0.51
- Male	46	13(28.3)	20(43.4)	13(28.3)	0.5/0.5
- Female	44	11(25.0)	21(47.7)	12(27.3)	0.49/0.51
		I/D-Exon 8			Frequency
	n	I/I (%)	I/D(%)	D/D(%)	I/D
Mothers	90	17(18.9)	58(64.4)	15(16.7)	0.51/0.49
Fathers	90	14(15.6)	49(54.4)	27(30.0)	0.43/0.57
Offspring	90	21(23.3)	47(52.2)	22(24.5)	0.49/0.51
- Male	46	10(21.7)	26(56.6)	10(21.7)	0.5/0.5
- Female	44	11(25.0)	21(47.7)	12(27.3)	0.49/0.51

Table 3: Genotype mating outcomes for the HLA-G polymorphisms in control and pre-eclampsia trios.

Mother	Father	Offspring	mt/mnt/pt/pnt	93***	Exon 8****
AA	AA	AA	AAAA	7	4
aa	aa	aa	aaaa	6	3
AA	aa	Aa	AAaa	6	7
aa	AA	Aa	aaAA	8	2
AA	Aa	AA	AAAa	4	1
AA	Aa	Aa	AAaA	2	5
aa	Aa	aa	aaaA	4	8
aa	Aa	Aa	aaAa	3	2
Aa	AA	AA	AaAA	7	7
Aa	AA	Aa	aAAA	12	1
Aa	aa	aa	aAaa	4	3
Aa	aa	Aa	Aaaa	1	14
Aa	Aa	AA	AaAa	6	9
Aa	Aa	Aa	AaaA*	0	10
			aAAa*	3	0
			(Aa)**	6	6
Aa	Aa	aa	aAaA	11	8
total				90	90

mt/mnt/pt/pnt = maternally transmitted / maternally non-transmitted / paternally transmitted / paternally non-transmitted. * Allele transmitted assigned from haplotype analysis, ** not possible to determine allele transmitted, ***for C/T-93 matings, A = C-93, a = T-93, ****for I/D-E8 matings, A = I-E8, a = D-E8

Table 4: Relative risk of foetal, maternal and parent of origin effects in a log linear model

	4-factor model		Stepwise reduced model[1]	
Risk Factor	Relative risks			
	C-93	I-E8	C-93	I-E8
1 or 2 alleles in offspring	0.38*	0.43	0.29**	0.39*
1 or 2 alleles in mother	0.85	1.17	----	----
maternal origin	0.70	4.03**	----	4.68***
paternal origin	1.59	0.88	2.12*	----
Chi-square improvement in fit [2]	14.6	23.8	12.9	24.5
overall p-value	0.006	0.0001	0.002	0.000005

* significance at the 5% level
** significance at the 1% level
*** significance at the 0.1% level
[1] The model was reduced by stepwise elimination of parameters whose significance was greater than 0.10.
[2] Compared to a model where none of the four factors shown are fitted i.e. only a term for mating-type stratification is fitted as it is for all models here.

Table 5: Comparisons within primigravida trios.

Maternal transmitted vs. paternal transmitted		
	C/T-93	p_1=0.009
	I/D-E8	p_1=0.000001
	93-E8 haplotype	p_3=0.000003
Maternal non-transmitted vs. paternal non-transmitted alleles		
	C/T-93	p_1=0.75
	I/D-E8	p_1=0.016
	93-E8 haplotype	p_3=0.028
Allele transmitted to offspring		
	C-93 vs. T-93 (transmission disequilibrium test)	p=0.062
	I-E8 vs. D-E8 (transmission disequilibrium test)	p=0.37
Maternal transmitted vs. non-transmitted alleles		
	C-93 vs. T-93 (transmission disequilibrium test)	p=0.032
	I-E8 vs. D-E8 (transmission disequilibrium test)	p=0.0005
Paternal transmitted vs. non-transmitted alleles		
	C-93 vs. T-93 (transmission disequilibrium test)	p=0.87
	I-E8 vs. D-E8 (transmission disequilibrium test)	p=0.01

Probability values (p) are presented with the numbers of degrees of freedom as a subscript.

Table 6: Extended haplotype transmission and frequency.

Haplotype	MT	MNT	PT	PNT	Frequency
polymorphic sites 31-93-107-110-E8					
a-a-a-a-b	17	30	36	25	0.321
a-b-a-a-a	41	18	18	23	0.298
a-a-a-a-a	6	11	10	19	0.137
a-b-a-a-b	6	16	14	9	0.134
a-a-a-b-b	2	5	2	2	0.032
a-a-b-a-a	4	2	2	2	0.030
a-a-a-b-a	3	0	0	1	0.012
a-a-b-a-b	1	1	0	1	0.009
a-b-b-a-b	0	1	1	1	0.009
a-b-b-a-a	2	0	0	0	0.006
a-b-a-b-b	1	0	1	0	0.006
a-b-a-b-a	1	0	0	0	0.003
a-a-b-b-b	0	0	0	1	0.003

37, 93, 107, 110, and E8 refer to the polymorphic sites in codon 31, 93, 107, 110 and E8. "a" and "b" represent the most common and least common allele respectively of each polymorphic site. MT/MNT/PT/PNT= maternally transmitted / maternally non-transmitted / paternally transmitted / paternally non-transmitted.

Table 7: Transmitted and non-transmitted HLA-G haplotypes to offspring in primigravida trios.

HLA-G Haplotype	Maternal transmitted haplotype	Maternal non-transmitted haplotype	Paternal transmitted haplotype	Paternal non-transmitted haplotype
C-93/I-E8	13 (0.16)	11 (0.13)	11 (0.13)	22 (0.26)
C-93/D-E8	21 (0.25)	38 (0.45)	40 (0.48)	29 (0.34)
T-93/D-E8	6 (0.07)	18 (0.22)	19 (0.22)	9 (0.11)
T-93/I-E8	44 (0.52)	17 (0.20)	14 (0.17)	24 (0.29)
n	84	84	84	84

Table 8: Transmitted and non-transmitted HLA-G haplotypes in trios.

Haplotype MT - PT		Offspring n	Haplotype T - NT		Mothers n	Fathers n
C-I	C-I	3	C-I	C-I	2	3
C-I	C-D	6	C-I	C-D	4	3
C-D	C-I	2	C-D	C-I	2	10
C-I	T-D	2	C-I	T-D	6	1
T-D	C-I	2	T-D	C-I	2	7
C-I	T-I	2	C-I	T-I	1	4
T-I	C-I	4	T-I	C-I	5	2
C-D	C-D	13	C-D	C-D	11	19
C-D	T-D	5	C-D	T-D	3	2
T-D	C-D	0	T-D	C-D	0	3
C-D	T-I	0	C-D	T-I	5	9
T-I	C-D	21	T-I	C-D	23	4
T-D	T-D	4	T-D	T-D	2	3
T-D	T-I	1	T-D	T-I	2	6
T-I	T-D	8	T-I	T-D	7	3
T-I	T-I	11	T-I	T-I	9	5
	n =	84			84	84

T: Haplotype transmitted to offspring, NT: Haplotype non-transmitted to offspring.
MT: Haplotype transmitted from mother to offspring, PT: Haplotype transmitted from father to offspring

Table 9: Genotype and allele distribution of the HLA-G C/T-93 and I/D-E8 polymorphisms in pre-eclampsia primigravida trios.

		C/T-93			Frequency	I/D-Exon 8			Frequency
Controls	n	C/C (%)	C/T (%)	T/T (%)	C/T	I/I (%)	I/D(%)	D/D(%)	I/D
Mothers	90	19(21.1)	50(55.6)	21(23.3)	0.49/0.51	17(18.9)	58(64.4)	15(16.7)	0.51/0.49
Fathers	90	34(37.8)	39(43.3)	17(18.9)	0.59/0.41	14(15.6)	49(54.4)	27(30.0)	0.43/0.57
Offspring	90	24(26.7)	41(45.5)	25(27.8)	0.49/0.51	21(23.3)	47(52.2)	22(24.5)	0.49/0.51
PE									
Mothers	79	30(37.9)	36(45.6)	13(16.5)	0.61/0.39	13(16.5)	47(59.5)	19(24.0)	0.46/0.54
Fathers	76	15(19.7)	48(63.2)	13(17.1)	0.51/0.49	19(25.0)	47(61.8)	10(13.2)	0.56/0.44
Offspring	82	18(22.0)	57(69.5)	7(8.5)	0.57/0.43	14(17.1)	55(67.1)	13(15.8)	0.51/0.49

Table 10: Transmitted and non-transmitted HLA-G haplotypes to offspring in control and pre-eclampsia trios.

HLA-G Haplotype	Maternal transmitted haplotype	Maternal non-transmitted haplotype	Paternal transmitted haplotype	Paternal non-transmitted haplotype
Control Trios				
C-93/I-E8	13 (0.16)	11 (0.13)	11 (0.13)	22 (0.26)
C-93/D-E8	21 (0.25)	38 (0.45)	40 (0.48)	29 (0.34)
T-93/D-E8	6 (0.07)	18 (0.22)	19 (0.22)	9 (0.11)
T-93/I-E8	44 (0.52)	17 (0.20)	14 (0.17)	24 (0.29)
n	84	84	84	84
PE Trios				
C-93/I-E8	5 (0.07)	13 (0.18)	12 (0.17)	11 (0.16)
C-93/D-E8	42 (0.60)	27 (0.39)	22(0.32)	24 (0.35)
T-93/D-E8	4 (0.06)	5 (0.07)	3(0.04)	11 (0.16)
T-93/I-E8	19 (0.27)	25 (0.36)	33(0.47)	22 (0.33)
n	70	70	70	68

Table 11: Genotype mating outcomes for the HLA-G polymorphisms in control and pre-eclampsia trios.

				Control Trios		PE Trios	
Mother	Father	Offspring	mt/mnt/pt/pnt	93[‡]	Exon 8[‡]	93[‡]	Exon 8[‡]
AA	AA	AA	AAAA	7	4	4	1
aa	aa	aa	aaaa	6	3	0	0
AA	aa	Aa	AAaa	6	7	8	2
aa	AA	Aa	aaAA	8	2	0	7
AA	Aa	AA	AAAa	4	1	7	5
AA	Aa	Aa	AAaA	2	5	9	4
aa	Aa	aa	aaaA	4	8	4	5
aa	Aa	Aa	aaAa	3	2	9	6
Aa	AA	AA	AaAA	7	7	4	0
Aa	AA	Aa	aAAA	12	1	7	11
Aa	aa	aa	aAaa	4	3	0	4
Aa	aa	Aa	Aaaa	1	14	5	4
Aa	Aa	AA	AaAa	6	9	2	6
Aa	Aa	Aa	AaaA*	0	10	6	3
			aAAa*	3	0	2	5
			(Aa)[†]	6	6	6	7
Aa	Aa	aa	aAaA	1-1	8	2	3

mt/mnt/pt/pnt = maternally transmitted / maternally non-transmitted / paternally transmitted / paternally non-transmitted. [‡]For C/T-93 matings, A = C-93, a = T-93, [‡]for I/D-E8 matings, A = I-E8, a = D-E8. *Allele transmitted assigned from haplotype analysis, [†] not possible to determine allele transmitted.

Table 12: Comparisons between Control and pre-eclampsia trios

	Mothers	Fathers	Offspring
Allele frequency			
C/T-93	p_1= 0.03	p_1= 0.14	p_1= 0.18
I/D-E8	p_1= 0.37	p_1= 0.02	p_1= 0.83
93-E8 haplotype frequency	p_3=0.03	p_3=0.008	p_3=0.03
Genotype distribution			
C/T-93	p_2= 0.05	p_2= 0.02	p_2= 0.001
I/D-E8	p_2= 0.49	p_2= 0.02	p_2= 0.14

Deviation from Hardy-Weinberg equilibrium

	Control Offspring	Control Mothers	Control Fathers	PE Offspring	PE Mothers	PE Fathers
C/T-93	p_1=0.40	p_1=0.29	p_1=0.34	p_1=0.0002	p_1=0.69	p_1=0.021
I/D-E8	p_1=0.67	p_1=0.006	p_1=0.29	p_1=0.002	p_1=0.08	p_1=0.027

Parental transmission to offspring

	MT	MNT	PT	PNT
C/T-93	p_1=0.0007	p_1=0.88	p_1=0.13	p_1=0.25
I/D-E8	p_1=0.00006	p_1=0.009	p_1=0.00002	p_1=0.44
93-E8 haplotype	p_3=0.0002	p_3=0.02	p_3=0.00003	p_3=0.43

MT = maternally transmitted, MNT = maternally non-transmitted
PT = paternally transmitted, PNT = paternally non-transmitted
Probability values (p) are presented with the numbers of degrees of freedom as a subscript.

Table 13: Comparisons within control trios and within pre-eclampsia trios.

a) Comparison of transmitted and non-transmitted alleles		Controls	PE
Heterozyogte vs. homozygote mating outcome			
	C/T-93	p_1=0.256	p_1=0.002
	I/D-E8	p_1=0.317	p_1=0.014
Allele transmitted to offspring			
	C-93 v. T-93 (TDT)	table 5	p=0.49
	I-E8 v. D-E8 (TDT)	table 5	p=0.77
Maternal transmitted vs. non-transmitted alleles			
	C-93 v. T-93 (TDT)	table 5	p=0.65
	I-E8 v. D-E8 (transmission disequilibrium test)	table 5	p=0.09
Paternal transmitted vs. non-transmitted alleles			
	C-93 v. T-93 (transmission disequilibrium test)	table 5	p=0.60
	I-E8 v. D-E8 (transmission disequilibrium test)	table 5	p=0.24
b) Test of difference between parent of origin			
Maternal transmitted vs. paternal transmitted			
	C-93 v. T-93	table 5	p_1=0.03
	I-E8 v. D-E8	table 5	p_1=0.0007
	93/E8 haplotypes	table 5	p_3=0.005
Maternal non-transmitted vs. paternal non-transmitted alleles			
	C-93 v. T-93	table 5	p_1=0.5
	I-E8 v. D-E8	table 5	p_1=0.49
	93/E8 haplotypes	table 5	p_3=0.43

Probability values (p) are presented with the numbers of degrees of freedom as a subscript.

Table 14: Transmitted and non-transmitted HLA-G haplotypes in control and pre-eclampsia trios.

HLA-G Haplotype		Control Offspring	PE Offspring	HLA-G Haplotype		Control Mothers	Control Fathers	PE Mothers	PE Fathers
MT	PT	n	n	T	NT	n	n	n	n
C-I	C-I	3	1	C-I	C-I	2	3	0	2
C-I	C-D	6	3	C-I	C-D	4	3	3	3
C-D	C-I	2	2	C-D	C-I	2	10	11	1
C-I	T-D	2	0	C-I	T-D	6	1	0	5
T-D	C-I	2	3	T-D	C-I	2	7	1	1
C-I	T-I	2	1	C-I	T-I	1	4	2	2
T-I	C-I	4	4	T-I	C-I	5	2	1	7
C-D	C-D	13	8	C-D	C-D	11	19	16	8
C-D	T-D	5	3	C-D	T-D	3	2	2	1
T-D	C-D	0	1	T-D	C-D	0	3	0	0
C-D	T-I	0	22	C-D	T-I	5	9	13	12
T-I	C-D	21	9	T-I	C-D	23	4	8	13
T-D	T-D	4	0	T-D	T-D	2	3	1	0
T-D	T-I	1	0	T-D	T-I	2	6	2	1
T-I	T-D	8	0	T-I	T-D	7	3	2	5
T-I	T-I	11	6	T-I	T-I	9	5	8	7
	n =	84	63			84	84	70	68

MT: haplotype transmitted from mother to offspring, PT: haplotype transmitted from father to offspring

T: haplotype transmitted to offspring, NT: haplotype non-transmitted to offspring.

Table 15: Relative risk of foetal, maternal and parent of origin effects in a log linear model

Risk Factor	Relative risks	
	PE	
	C-93	I-E8
1 or 2 alleles in offspring	3.51*	1.7
1 or 2 alleles in mother	0.66	0.98
maternal origin	1.12	0.59
paternal origin	0.74	1.11

* significance at the 5% level

Table 16: Genotype and allele distribution of the HLA-G C/T-93 and I/D-E8 polymorphisms in recurrent miscarriage couples.

		C/T-93			Frequency
	n	C/C	C/T	T/T	C/T
Females	22	13	7	2	0.75/0.25
Males	20	6	12	2	0.6/0.4

		I/D-Exon 8			Frequency
	n	I/I (%)	I/D(%)	D/D(%)	I/D
Females	22	3	11	8	0.39/0.61
Males	21	5	12	3	0.55/0.45

	C-93/I-E8	C-93/D-E8	T-93/D-E8	T-93/I-E8
Females	7	25	1	9
Males	7	17	1	15

Table 17: HLA-G haplotypes in recurrent miscarriage couples.

Couple no.	Female partner HLA_G haplotype	Male partner HLA-G haplotype
1	t-i / c-d	t-i / c-d
2	c-i / c-d	c-i / c-d
3	c-i / c-d	t-i / c-d
4	c-i / c-d	t-i / c-d
5	c-d / c-d	t-i / c-d
6	c-i / c-d	c-i / c-i
7	c-d / c-d	c-i / t-i
8	c-i / t-i	t-d / c-i
9	t-i / c-d	t-i / t-i
10	t-i / t-i	t-i / c-d
11	c-d / c-d	c-i / c-i
12	c-d / c-d	t-i / c-d
13	t-i / t-i	t-i / c-d
14	t-d / c-i	c-d / c-d
15	c-d / c-d	t-i / t-i
16	c-i / c-d	t-i / c-d
17	c-d / c-d	c-d / c-d
18	c-d / c-d	c-d / c-d
19	t-i / c-d	t-i / c-d
20	t-i / c-d	t-i / c-d

Table 18: Transmitted and non-transmitted HLA-G haplotypes (extended genotypes) in first and second offspring of normal mothers

haplotypes	CMT / MNT	CFT / FNT	first offspring		second offspring
C-I / C-D	2	2	2		0
C-D/ C-I	1	2	5		5
C-I / T-D	1	0	0		0
T-D / C-I	0	1	1		1
C-I / T-I	2	5	3		1
T-I / C-I	4	0	0		0
C-D/ T-D	3	1	2		0
T-D / C-D	5	3	3		2
C-D / T-I	6	3	0		5
T-I / C-D	2	2	1		5
T-D / T-I	3	3	4		3
T-I / T-D	2	6	6		6
C-I / C-I	0	0	0		0
C-D / C-D	12	14	14		11
T-D / T-D	1	1	1		0
T-I / T-I	4	5	5		8
T-I / T-I	4	5	5		8

MT: haplotype transmitted from mother to offspring, PT: haplotype transmitted from father to offspring
T: haplotype transmitted to offspring, NT: haplotype non-transmitted to offspring.

Table 19: Transmitted and non-transmitted HLA-G extended genotypes in first and second offspring of primigravida pre-eclampsia mothers

	first offspring pre-eclampsia	second offspring normal pregnancy	mother	father	
	MT / PT	MT / PT			
1	C-D / C-D	C-D / C-D	C-I / C-D	C-D / C-D	
2	T-I/ C-D	C-D / C-D	C-D / T-I	C-I / C-D	
3	T-I/ C-D	T-I/ C-D	T-I/ T-I	C-D / T-I	
4	C-I / C-D	C-D / C-D	C-I / C-D	C-D / T-I	
5	C-D / T-I*	C-D / T-I *	C-D / C-D	C-D / T-I	
6	C-D / T-I*	C-D / T-I*	C-D / T-D	T-I / T-I	
7	C-D / T-I*	C-D / T-I*	C-D / C-D	C-I / T-I	
8	T-D / C-I	T-D / C-I	T-D / C-I	C-I / C-I	
9	T-I/ C-D	T-I/ C-D	T-I / T-I	C-D / T-I	

MT: haplotype transmitted from mother to offspring, PT: haplotype transmitted from father to offspring

CLAIMS

1. A method for diagnosing susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility comprising the steps of:

a) obtaining a fluid and/or tissue sample from a female and/or male and/or foetus; and either

b) determining the sequence of all or part of the HLA-G nucleic acid, and/or HLA-G linked nucleic acid; or

c) detecting variant forms of all or part of the HLA-G protein, and/or proteins encoded by HLA-G linked genes or:

d) measuring the functional activity of all or part of the HLA-G encoding protein and/or proteins encoded by HLA-G linked genes or:

e) measuring the size and /or level of all or part of HLA-G mRNA or mRNA transcribed from HLA-G linked genes or:

f) measuring the size and /or level of all or part of HLA-G protein and/or protein encoded by HLA-G linked genes or:

g) quantifying cells or molecules whose concentration changes as a result of HLA-G action; and

h) comparing any of the parameters b) to g) with those of a female and/or male and/or foetus of a normal pregnancy and/or a pregnancy with pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related fertility outcome.

2. A method as claimed in claim 1 wherein the HLA-G nucleic acid is analysed for the presence of the C and/or T allele of codon 93 in exon 3 and/or the insertion and/or deletion allele of exon 8.

3. A method as claimed in claim 1 wherein the effect of one or more of the HLA-G sequence variants on the functional activity of HLA-G and / or on the size and /or level of all or part of HLA-G mRNA and/or its encoded polypeptide is measured.

4. A method as claimed in claim 1 or 2 wherein all or part of any HLA-G sequence and/or HLA-G linked sequences is amplified, preferably by a method or combination of methods selected from the polymerase chain reaction, nucleic acid sequence based amplification, self sustained sequence replication, transcription-mediated amplification, strand displacement amplification, and the ligase chain reaction.

5. A method as claimed in claim 1-4 wherein comparing of one or more variants identified is performed by association and/or linkage analysis and/or transmission analysis.

6. A method as claimed in any preceding claim wherein all or part of the HLA-G sequence is cloned into a vector.

7. A method as claimed in any preceding claim wherein all or part of the nucleic acid sequence is identified by a method or combination of methods selected from DNA sequencing, glycosylase mediated polymorphism detection, restriction fragment length polymorphism analysis, enzymatic or chemical cleavage analysis, hybridisation to DNA and /or RNA probes and /or DNA probe arrays and/or allele specific DNA and /or RNA probes, allele specific amplification analysis, electrophoretic mobility analysis and 5' nuclease assay analysis.

8. A method as claimed in any preceding claim wherein all or part of HLA-G and /or all or part of one or more variants thereof is expressed as a polypeptide *in vitro* and/or in a prokaryotic and / or eukaryotic cell.

9. A method as claimed in claim 1 wherein the cells of step (g) are blood mononuclear cells and / or T cell and /or natural killer cell subsets thereof and/or HLA-G expressing cells.

10. A method as claimed in any preceding claim wherein the activity of HLA-G and/or any combination of variants thereof and/or blood mononuclear cells and /or a subset of such cells, selected from T cells and/or natural killer cells, is measured by one or more of the following procedures:

(a) measuring the interaction of HLA-G and /or variants thereof with blood mononuclear cells and/or subsets thereof by assessing one or more of the following with respect to HLA-G expressing cells and /or blood mononuclear cells; cell proliferation, transformation, cytotoxic response, surface marker expression, cytokine production, conjugate formation and target specificity,

(b) measuring the size and / or level of all or part of HLA-G mRNA and/or its encoded polypeptide,

(c) measuring the peptide binding capability of all or part of HLA-G and /or variants thereof,

(d) measuring the binding capability of all or part of the HLA-G and /or variants thereof to a HLA-G receptor,

(e) measuring one or more molecules whose level is altered as a result of the interaction of the HLA-G and /or variants thereof and /or cells expressing HLA-G with blood mononuclear cells,

(f) measuring the expression levels of one or more genes and/or proteins in the HLA-G expressing cells.

11. A method as claimed in any preceding claim wherein blood mononuclear cells and/or subsets thereof and/or HLA-G and/or HLA-G linked variants thereof and/or cells expressing all or part of the variants fully and/or partially matching a female and/or male and/or foetus are selected from a test panel.

12. A method as claimed in any preceding claim wherein the HLA-G is partially or fully purified from a cell expressing HLA-G.

13. A method as claimed in any preceding claim wherein the HLA-G is detected by immunoassay using one or more antibodies specific for HLA-G and/or variants thereof.

14. A method as claimed in any preceding claim wherein all or part of the HLA-A, HLA-B, HLA-C, HLA-E, HLA-F and HLA-H genes are analysed in the female and/or male and/or foetus.

15. A method as claimed in claim 1 wherein the molecules of step (g) are selected from IL-1 beta, IL-2, IL-3, IL-4, IL-6, IL-10 and tumour necrosis factor-alpha, or trophoblast specific markers selected from cytokeratins, pregnancy specific glycoprotein 1, human chorionic gonadotrophin and human placental lactogen.

16. A method for screening for agents which can potentially be used as diagnostic indicators and/or drug targets for pre-eclampsia, miscarriage, miscarriage-related infertility and intrauterine growth retardation by:

a) measuring the expression level of one or more genes and/or proteins in HLA-G expressing cells and/or blood mononuclear cells and/or T cell and/or natural killer cells subsets thereof following interaction with HLA-G and/or HLA-G expressing cells;

b) comparing the expression level identified in step a) with the expression level in HLA-G expressing cells and/or the blood mononuclear cells and/or T cell and/or natural killer cell subsets thereof following interaction with HLA-G and/or HLA-G expressing cells in normal pregnancy and/or pre-eclampsia pregnancy and/or intrauterine growth retardation pregnancy and/or miscarriage pregnancy and/or miscarriage-related infertility.

17. A method as claimed in claim 10 or 16 wherein gene expression and/or protein expression is measured by any one or combination of methods selected from hybridisation between cDNA and/or RNA from the cells and DNA probes and/or RNA probes and/or nucleic acid probe arrays, quantitative amplification methods, reverse transcriptase - polymerase chain reaction (RT-PCR), 5' nuclease assay, ribonuclease protection assay and S1 nuclease assay, one dimensional and/or two dimensional gel electrophoresis and staining of proteins, detection of one or more proteins using, enzyme linked immunosorbent assays (ELISA), radioimmunoassays (RIA), protein truncation test (PTT), immunoradiometric assays (IRMA) and immunoenzymatic assays (IEMA), sandwich assays and Western blotting using monoclonal and/or polyclonal antibodies.

18. A pharmaceutical composition comprising a pharmaceutically effective amount of HLA-G and/or cells expressing HLA-G and/or one or more peptides which binds to HLA-G, blood mononuclear cells from a donor and/or test panel known to interact with HLA-G variants, cytokines and any combination thereof including IL-1 beta, IL-2, IL-3, IL-4, IL-6, IL-10 and tumour necrosis factor-alpha and/or

inhibitors of cytokines and/or tumour necrosis factor alpha and/or derivatives of cytokines and/or tumour necrosis factor-alpha, optionally with pharmaceutically-acceptable carriers or excipients.

19. A method for screening potential pre-eclampsia and eclampsia and intrauterine growth retardation and miscarriage and miscarriage-related infertility therapeutic agents selected from:

a) identifying agents which alter the expression of HLA-G;

b) identifying agents which alter the activity of HLA-G;

c) identifying agents which mimic the action of HLA-G;

d) identifying agents which bind to HLA-G;

e) identifying peptides which bind to HLA-G;

f) identifying agents which bind to HLA-G receptors;

g) identifying expressed genes using DNA probe arrays in a cellular background in HLA-G expressing cells and / or blood mononuclear cells interacting with HLA-G and / or cells expressing HLA-G interacting with blood mononuclear cells;

h) identifying expressed proteins using mass spectrometry methods in HLA-G expressing cells and / or blood mononuclear cells interacting with HLA-G and / or cells expressing HLA-G interacting with blood mononuclear cells;

i) identifying expressed proteins using mass spectrometry methods in HLA-G expressing cells and / or blood mononuclear cells interacting with HLA-G and / or cells expressing HLA-G interacting with blood mononuclear cells;

j) screening sperm and/or semen and/or female reproductive tissue for agents:

i) which alter the expression of HLA-G in fertilised eggs and/or embryos;

ii) which alter the cell cleavage rate of fertilised eggs and/or embryos;

iii) which induce cellular factors in cells in culture and/or cells *in vivo* that alter the cell cleavage rate of fertilised eggs and/or embryos.

20. A method for the prevention of pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility selected from:

a) treatment of a female with all or part of a pharmaceutically effective amount of an effective HLA-G and /or peptides which bind to HLA-G and / or cells expressing HLA-G;

b) treatment of a female with all or part of a pharmaceutically effective amount of molecules or inhibitors of molecules whose level or activity is directly or indirectly altered by HLA-G action;

c) treatment of a female with all or part of a pharmaceutically effective amount of molecules which inhibit the interaction between HLA-G and one or more of its receptors;

d) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters HLA-G expression:

e) treatment of a female with all or part of a pharmaceutically effective amount of an agent which alters HLA-G related blood mononuclear cell activity;

f) treatment of a female with all or part of a pharmaceutically effective amount of an agent which mimics all or part of HLA-G action;

g) treatment of a female with blood mononuclear cells that recognise foetal and / or self HLA-G;

h) treatment of a female with HLA-G and / or cells expressing HLA-G or variants thereof;

i) treatment of a female with one or more antibodies which bind to HLA-G and / or cells expressing HLA-G and / or any receptor for HLA-G;

j) introduction of one or more variants of the HLA-G gene and /or its receptor into a female and / or male;

k) introduction of an inhibitor of expression of the HLA-G gene and/or its receptor into a female and/or male;

l) inactivation of one or more variants of the HLA-G gene and/or its receptor in a female and/or male.

21. A method for improving pregnancy success selected from:

a) pre-treating the female with sperm and/or attenuated forms thereof, and/or semen and/or fractions thereof from a male with a known HLA-G genotype, prior to mating with a male of a different HLA-G genotype, and/or in vitro fertilisation using sperm from a male of a different HLA-G genotype and/or embryo transfer where the male HLA-G is of a different HLA-G genotype;

b) mixing sperm of a known HLA-G genotype with sperm and/or attenuated forms thereof, and/or semen and/or fractions thereof from a male with a different HLA-G genotype prior to in vitro fertilisation.

22. A method as claimed in claim 21 wherein fertility and / or pregnancy outcome are improved by selection of male and / or female partners and / or sperm and / or ova and / or recipients of fertilised eggs and / or zygotes / and / or embryos so that (a) their HLA-G and /or HLA genotypes and /or serotypes or (b) the activity of their HLA-G and / or blood mononuclear cells interacting with HLA-G are indicative of normal pregnancy outcomes and / or not associated with pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility.

23. A test kit for the diagnosis of susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility or for monitoring progress of pregnancy comprising:

a) oligonucleotide primers for amplification of all or part of the HLA-G gene and /or HLA-G linked DNA;

b) amplification reagents for amplification of genomic DNA and / or RNA segments, selected from a DNA / RNA polymerase, a reverse transcriptase, the deoxyribonucleotides dATP, dCTP, dGTP, dTTP and dUTP, and /or ribonucleotides ATP, CTP, GTP, TTP and UTP, and reaction buffer;

c) reagents for identifying sequence variants in DNA and / or RNA;

d) control DNA and /or RNA.

24. Use of a DNA sequence selected from any one of Sequence I.D.s 1 to 21 for diagnosis of susceptibility to or in a test kit for the diagnosis of susceptibility to normal pregnancy, pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility, for monitoring progress of pregnancy, for use in the manufacture of a medicament, in a method for screening potential therapeutic agents, in a method for screening for potential diagnostic indicators and/or drug targets, in a method for improving pregnancy success or in a method for the prevention of pre-eclampsia and/or eclampsia and/or intrauterine growth retardation and/or susceptibility to miscarriage and/or miscarriage-related infertility, for monitoring progress of pregnancy.

25. A method for induction of tolerance in a host to a non-self tissue which comprises administering HLA-G and /or HLA-G loaded with peptides from the non-self tissue and /or HLA-G expressing cells derived from or related to the non-self tissue, and/or a non-self tissue bearing an introduced HLA-G so that HLA-G is expressed in all or part of the tissue.

26. A method for the treatment of autoimmune disease which comprises administering HLA-G and /or HLA-G loaded with peptides from a self and/or non-self tissue and / or with specific autoimmune antigen and /or HLA-G expressing cells from a self and/or non-self tissue and/or a self and/or self tissue bearing an introduced HLA-G gene so that HLA-G is expressed in all or part of the tissue.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/IE 99/00012

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12Q1/02 C12N15/12 G01N33/50 G01N33/564
A61K38/17 A61K35/14 A61K39/395 //C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	OBER C ET AL: "Population genetic studies of HLA-G: allele frequencies and linkage disequilibrium with HLA-A " JOURNAL OF REPRODUCTIVE IMMUNOLOGY, vol. 32, no. 2, December 1996, page 111-23 XP002105897 see page 120, paragraph 2 - paragraph 3 ---	1-24
A	OBER C ET AL: "HLA-G polymorphisms: neutral evolution or novel function" JOURNAL OF REPRODUCTIVE IMMUNOLOGY, vol. 36, no. 1-2, November 1997, page 1-21 XP002105898 see the whole document --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
15 June 1999	06/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office. P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	HUMPHREY K ET AL: "HLA-G deletion polymorphism and pre-eclampsia/eclampsia" BRITISH JOURNAL OF OBSTETRICS AND GYNAECOLOGY, vol. 102, no. 9, September 1995, pages 707-10, XP002105899 see the whole document	1-24
A	MCMASTER M ET AL: "Immunology of human pregnancy" CURRENT PROBLEMS IN OBSTETRICS, GYNECOLOGY AND FERTILITY, vol. 21, no. 1, January 1998, pages 6-23, XP002105900 see page 9 - page 13	1-24
A	KARHUKORPI J ET AL: "HLA-G polymorphism in Finnish couples with recurrent spontaneous miscarriage" BRITISH JOURNAL OF OBSTETRICS AND GYNAECOLOGY, vol. 104, no. 10, October 1997, pages 1212-14, XP002105901 see the whole document	1-24
A	STEFFENSEN R ET AL: "HLA-G PCR-RPFL typing of woman with multiple spontaneous abortions" HUMAN BIOLOGY, vol. 47, no. 1-2, 1996, page 147 XP002105902 see abstract P798	1-24
A	HENNESSY A ET AL: "Reduced expression of immunosupressor genes in preeclampsia (PE)" JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY, vol. 9, September 1997, page 330A XP002105903 see abstract A1376.	1-24
A	HARA N ET AL: "Altered expression of human leukocyte antigen G (HLA-G) on extravillous trophoblasts in preeclampsia" AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY, vol. 36, no. 6, December 1996, pages 349-58, XP002105904 see abstract on 349	1-24

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/IE 99/00012

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MAIN E ET AL: "Nulliparous preeclampsia (PE) is associated with placental expression of a variant allele of the new histocompatibility gene: HLA-G" AMERICAN JOURNAL OF OBSTETRICS AND GYNECOLOGY, vol. 170, no. 1(part 2) , 1994, page 289 XP002105905 see the whole document	1-26
A	CAROSELLA E D ET AL: "HLA-G revisited" IMMUNOLOGY TODAY, vol. 17, no. 9, 1 September 1996, page 407-409 XP004034736 see the whole document	1-24
A	WO 96 31604 A (UNIV CALIFORNIA) 10 October 1996 see the whole document	25,26
A	WO 95 31472 A (UNIV TEXAS) 23 November 1995	25,26
A	FR 2 717 498 A (COMMISSARIAT ENERGIE ATOMIQUE) 22 September 1995 see the whole document	25,26

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IE 99/00012

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 20, 21, 25 and 26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: partially 25 and 26
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: partially 25 and 26.

Method claims 25 and 26 (also see Remark) have been searched only insofar as the methods relate to susceptibility to normal pregnancy, pre-eclampsia and/ or eclampsia and/ or intrauterine growth retardation and/ or susceptibility to miscarriage and/ or miscarriage-related infertility.

INTERNA NAL SEARCH REPORT

Information on patent family members

[redacted] nal Application No
PCT/IE 99/00012

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9631604 A	10-10-1996	AU 696118 B	03-09-1998
		AU 5256896 A	23-10-1996
		CA 2213620 A	10-10-1996
		EP 0819171 A	21-01-1998
		JP 11503320 T	26-03-1999
WO 9531472 A	23-11-1995	AU 688914 B	19-03-1998
		AU 2513395 A	05-12-1995
		CA 2190576 A	23-11-1995
		EP 0759930 A	05-03-1997
		JP 10501690 T	17-02-1998
FR 2717498 A	22-09-1995	EP 0677582 A	18-10-1995
		US 5856442 A	05-01-1999

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